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(54) Title: A GENE SWITCH COMPRISING AN ECDYSONE RECEPTOR

(57) Abstract

The invention relates to an insect steroid receptor protein which is capable of acting as a gene switch which is responsive to a chemical inducer enabling external control of the gene.

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A gene switch comprising an ecdysone receptor

The present invention relates to the identification and characterisation of insect steroid receptors from the Lepidoptera species *Heliothis virescens*, and the nucleic acid encoding therefor. The present invention also relates to the use of such receptors, and such nucleic acid, particularly, but not exclusively, in screening methods, and gene switches.

By gene switch we mean a gene sequence which is responsive to an applied exogenous chemical inducer enabling external control of expression of the gene controlled by said gene sequence.

Lipophilic hormones such as steroids induce changes in gene expression to elicit profound effects on growth, cellular differentiation, and homeostasis. These hormones recognise intracellular receptors that share a common modular structure consisting of three main functional domains: a variable amino terminal region that contains a transactivation domain, a DNA binding domain, and a ligand binding domain on the carboxyl side of the molecule. The DNA binding domain contains nine invariant cysteines, eight of which are involved in zinc coordination to form a two-finger structure. In the nucleus the hormone-receptor complex binds to specific enhancer-like sequences called hormone response elements (HREs) to modulate transcription of target genes.

The field of insect steroid research has undergone a revolution in the last three years as a result of the cloning and preliminary characterisation of the first steroid receptor member genes. These developments suggest the time is ripe to try to use this knowledge to improve our tools in the constant fight against insect pests. Most of the research carried out on the molecular biology of the steroid receptor superfamily has been on *Drosophila melanogaster* (Diptera), see for example International Patent Publication No WO91/13167, with some in *Manduca* and *Galleria* (Lepidoptera).

It has been three decades since 20-hydroxyecdysone was first isolated and shown to be involved in the regulation of development of insects. Since then work has been carried out to try to understand the pathway by which this small hydrophobic molecule regulates a number of activities. By the early 1970s, through the studies of Clever and Ashburner, it was clear that at least in the salivary glands of third instar *Drosophila* larvae, the application of ecdysone lead to the reproducible activation of over a hundred genes. The ecdysone receptor in this pathway is involved in the regulation of two classes of genes: a small class (early genes) which are induced by the ecdysone receptor and a large class (late genes) which are repressed by the ecdysone receptor. The early class of genes are thought to have two functions reciprocal to those of the ecdysone receptor; the repression of the early transcripts and the induction of late gene transcription. Members of the early genes so far isolated and characterised belong to the class of molecules with characteristics similar to known

transcription factors. They are thus predicted to behave as expected by the model of ecdysone action (Ashburner, 1991). More recently, the early genes E74 and E75 have been shown to bind both types of ecdysone inducible genes (Thummel et al., 1990; Segraves and Hogness, 1991), thus supporting their proposed dual activities. It should be noted however, that the activation of a hierarchy of genes is not limited to third instar larvae salivary glands, but that the response to the ecdysone peak at the end of larval life is observed in many other tissues, such as the imaginal disks (i.e. those tissues which metamorphose to adult structures) and other larval tissues which histolyse at the end of larval life (eg. larval fat body). The model for ecdysone action as deduced by studying the third instar chromosome puffing may not apply to the activation of ecdysone regulated genes in adults. In other words, the requirement for other factors in addition to the active ecdysone receptor must be satisfied for correct developmental expression (e.g. the *Drosophila* yolk protein gene expression in adults is under control of doublesex, the last gene in the sex determination gene hierarchy).

The ecdysone receptor and the early gene E75 belong to the steroid receptor superfamily. Other *Drosophila* genes, including ultraspiracle, tailless, sevenup and FTZ-FI, also belong to this family. However, of all these genes only the ecdysone receptor is known to have a ligand, and thus the others are known as orphan receptors. Interestingly, despite the ultraspiracle protein ligand binding region sharing 49% identity with the vertebrate retinoic X receptor (RXR) ligand binding region (Oro et al., 1990), they do not share the same ligand (i.e. the RXR ligand is 9-cis retinoic acid) (Heymann et al., 1992 and Mangelsdorf et al., 1992). All the *Drosophila* genes mentioned are involved in development, ultraspiracle for example, is required for embryonic and larval abdominal development. The protein products of these genes all fit the main features of the steroid receptor superfamily (Evans, 1988; Green and Chambon, 1988, Beato, 1989) i.e. they have a variable N terminus region involved in ligand independent transactivation (Domains A and B), a highly conserved 66-68 amino acid region which is responsible for the binding of DNA at specific sites (Domain C), a hinge region thought to contain a nuclear translocation signal (Domain D), and a well conserved region containing the ligand binding region, transactivation sequences and the dimerisation phase (Domain E). The last region, domain F, is also very variable and its function is unknown.

Steroid receptor action has been elucidated in considerable detail in vertebrate systems at both the cellular and molecular levels. In the absence of ligand, the receptor molecule resides in the cytoplasm where it is bound by Hsp90, Hsp70, and p59 to form the inactive complex (Evans, 1988). Upon binding of the ligand molecule by the receptor a conformational change takes place which releases the Hsp90, Hsp70 and p59 molecules, while exposing the nuclear translocation signals in the receptor. The ligand dependent conformational change is seen in the ligand binding domain of both progesterone and retinoic acid receptors (Allan et

al., 1992a). This conformational change has been further characterised in the progesterone receptor and was found to be indispensable for gene transactivation (Allan et al., 1992b). Once inside the nucleus the receptor dimer binds to the receptor responsive element at a specific site on the DNA resulting in the activation or repression of a target gene. The receptor responsive elements usually consist of degenerate direct repeats, with a spacer between 1 and 5 nucleotides, which are bound by a receptor dimer through the DNA binding region (Domain C).

Whereas some steroid hormone receptors are active as homodimers others act as heterodimers. For example, in vertebrates, the retinoic acid receptor (RAR) forms heterodimers with the retinoic X receptor (RXR). RXR can also form heterodimers with the thyroid receptor, vitamin D receptor (Yu et al., 1991; Leid et al., 1992) and peroxisome activator receptor (Kliewer et al., 1992). Functionally the main difference between homodimers and heterodimers is increased specificity of binding to specific response elements. This indicates that different pathways can be linked, co-ordinated and modulated, and more importantly this observation begins to explain the molecular basis of the pleotropic activity of retinoic acid in vertebrate development (Leid et al., 1992b). Similarly, the *Drosophila* ultraspiracle gene product was recently shown to be capable of forming heterodimers with retinoic acid, thyroid, vitamin D and peroxisome activator receptors and to stimulate the binding of these receptors to their target responsive elements (Yao et al., 1993). More significantly, the ultraspiracle gene product has also been shown to form heterodimers with the ecdysone receptor, resulting in cooperative binding to the ecdysone response element and capable of rendering mammalian cells ecdysone responsive (Yao et al., 1992). The latter is of importance since transactivation of the ecdysone gene alone in mammalian cells fails to elicit an ecdysone response (Koelle et al., 1991), therefore suggesting that the ultraspiracle gene product is an integral component of a functional ecdysone receptor (Yao et al., 1992). It is possible that the ultraspiracle product competes with other steroid receptors or factors to form heterodimers with the ecdysone receptor. Moreover it remains to be investigated if ultraspiracle is expressed in all tissues of the *Drosophila* larvae. Despite ultraspiracle being necessary to produce a functional ecdysone receptor, the mechanism by which this activation takes place is as yet undetermined.

We have now isolated and characterised the ecdysone steroid receptor from *Heliothis virescens* (hereinafter HEcR). We have found that surprisingly unlike the *Drosophila* ecdysone steroid receptor (hereinafter DEcR), in reports to-date, HEcR can be induced by known non-steroidal inducers. It will be appreciated that this provides many advantages for the system.

Steroids are difficult and expensive to make. In addition, the use of a non-steroid as the inducer allows the system to be used in agrochemical and pharmaceutical applications, not

least because it avoids application of a steroid which is already present in insects and/or mammals. For example, it would not be feasible to use a gene switch in a mammalian cell which was induced by a naturally occurring steroidal inducer. It will also be appreciated that for environmental reasons it is advantageous to avoid the use of steroids as inducers.

According to one aspect of the present invention there is provided DNA having the sequence shown in Seq ID No. 2, wherein Seq ID No 2 gives the sequence for the HEcR.

According to another aspect of the present invention there is provided DNA having part of the sequence shown in Seq ID No. 2, which encodes for the HEcR ligand binding domain.

According to another aspect of the present invention there is provided DNA having part of the sequence shown in Seq ID No. 2, which encodes for the HEcR DNA binding domain.

According to yet another aspect of the present invention there is provided DNA having part of the sequence shown in Seq ID No. 2, which encodes for the HEcR transactivation domain.

According to a further aspect of the present invention there is provided DNA having part of the sequence shown in Seq ID No. 2, which encodes for the HEcR hinge domain.

According to a still further aspect of the present invention there is provided DNA having part of the sequence shown in Seq ID No. 2, which encodes for the HEcR carboxy terminal region.

According to one aspect of the present invention there is provided DNA having the sequence shown in Seq ID No. 3, wherein Seq ID No 3 gives the sequence for the HEcR.

According to another aspect of the present invention there is provided DNA having part of the sequence shown in Seq ID No. 3, which encodes for the HEcR ligand binding domain.

According to another aspect of the present invention there is provided DNA having part of the sequence shown in Seq ID No. 3, which encodes for the HEcR DNA binding domain.

According to yet another aspect of the present invention there is provided DNA having part of the sequence shown in Seq ID No. 3, which encodes for the HEcR transactivation domain.

According to a further aspect of the present invention there is provided DNA having part of the sequence shown in Seq ID No. 3, which encodes for the HEcR hinge domain.

According to a still further aspect of the present invention there is provided DNA having part of the sequence shown in Seq ID No. 3, which encodes for the HEcR carboxy terminal region.

According to one aspect of the present invention there is provided DNA having the sequence shown in Seq ID No. 4, wherein Seq ID No 4 gives the sequence for the HEcR.

According to another aspect of the present invention there is provided DNA having part of the sequence shown in Seq ID No. 4, which encodes for the HEcR ligand binding domain.

According to another aspect of the present invention there is provided DNA having part of the sequence shown in Seq ID No. 4, which encodes for the HEcR DNA binding domain.

According to yet another aspect of the present invention there is provided DNA having part of the sequence shown in Seq ID No. 4, which encodes for the HEcR transactivation domain.

According to a further aspect of the present invention there is provided DNA having part of the sequence shown in Seq ID No. 4, which encodes for the HEcR hinge domain.

According to a still further aspect of the present invention there is provided DNA having part of the sequence shown in Seq ID No. 4, which encodes for the HEcR carboxy terminal region.

As mentioned above, steroid receptors are eukaryotic transcriptional regulatory factors which, in response to the binding of the steroid hormone, are believed to bind to specific DNA elements and activate transcription. The steroid receptor can be divided into six regions, designated A to F, using alignment techniques based on shared homology with other members of the steroid hormone receptor superfamily. Krust et al identified two main regions in the receptor, C and E. Region C is hydrophilic and is unusual in its high content in cysteine, lysine and arginine.It corresponds to a DNA-binding domain, sometimes referred to as the "zinc finger". It is the DNA binding domain which binds to the upstream DNA of the responsive gene. Such upstream DNA is known as the hormone response element or HRE for short. Region E is hydrophobic and is identified as the hormone (or ligand) binding domain. Region E can be further subdivided into regions E1, E2 and E3.

The region D, which separates domains C and E is highly hydrophobic and is flexible. It is believe that communication between domains E and C involves direct contact between them through region D, which provides a hinge between the two domains. Region D is therefore referred to as the hinge domain.

The mechanism of the receptor appears to require it to interact with some element(s) of the transcription machinery over and above its interactions with the hormone and the hormone response element. N-terminal regions A and B perform such a function and are jointly known as the transactivation domain. The carboxy-terminal region is designated F.

The domain boundaries of the HEcR can be defined as follows:

DOMAIN	INTERVALS	
	base pairs	amino acids
Transactivating (A/B)	114-600	1-162
DNA Binding (C)	601-798	163-228
Hinge (D)	799-1091	229-326
Ligand Binding (E)	1092-1757	327-545
C-Terminal End (F)	1758-1844	546-577

The DNA binding domain is very well defined and is 66 amino acids long, thus providing good boundaries. The above intervals have been defined using the multiple alignment for the ecdysone receptors (Figure 5).

The present invention also includes DNA which shows homology to the sequences of the present invention. Typically homology is shown when 60% or more of the nucletides are common, more typically 65%, preferably 70%, more preferably 75%, even more preferably 80% or 85%, especially preferred are 90%, 95%, 98% or 99% or more homology.

The present invention also includes DNA which hybridises to the DNA of the present invention and which codes for at least part of the *Heliothis* ecdysone receptor transactivation domain, DNA binding domain, hinge domain, ligand binding domain and/or carboxy terminal region. Preferably such hybridisation occurs at, or between, low and high stringency conditions. In general terms, low stringency conditions can be defined as 3 x SCC at about ambient temperature to about 65°C, and high stringency conditions as 0.1 x SSC at about 65°C. SSC is the name of a buffer of 0.15M NaCl, 0.015M trisodium citrate. 3 x SSC is three time as strong as SSC and so on.

The present invention further includes DNA which is degenerate as a result of the genetic code to the DNA of the present invention and which codes for a polypeptide which is at least part of the *Heliothis* ecdysone receptor transactivation domain, DNA binding domain, hinge domain, ligand binding domain and/or carboxy terminal region.

The DNA of the present invention may be cDNA or DNA which is in an isolated form.

According to another aspect of the present invention there is provided a polypeptide comprising the *Heliothis* ecdysone receptor or a fragment thereof, wherein said polypeptide is substantially free from other proteins with which it is ordinarily associated, and which is coded for by any of the DNA of the present invention.

According to another aspect of the present invention there is provided a polypeptide which has the amino acid sequence of Seq ID No. 4 or any allelic variant or derivative thereof, wherein Seq ID No. 4 gives the amino acid sequence of the HEcR polypeptide.

According to another aspect of the present invention there is provided a polypeptide which has part of the amino acid sequence of Seq ID No. 4 or any allelic variant or derivative thereof, which sequence provides the HEcR ligand binding domain.

According to another aspect of the present invention there is provided a polypeptide which has part of the amino acid sequence of Seq ID No. 4 or any allelic variant or derivative thereof, which sequence provides the HEcR DNA binding domain.

According to yet another aspect of the present invention there is provided a polypeptide which has part of the amino acid sequence of Seq ID No. 4 or any allelic variant or derivative thereof, which sequence provides the HEcR transactivation domain.

According to a further aspect of the present invention there is provided a polypeptide which has the amino acid sequence of a part of Seq ID No. 4 or any allelic variant or derivative thereof, which sequence provides the HEcR hinge domain.

According to a still further aspect of the present invention there is provided a polypeptide which has the amino acid sequence of a part of Seq ID No. 4 or any allelic variant or derivative thereof, which sequence provides the HEcR carboxy terminal region.

For the avoidance of doubt, spliced variants of the amino acid sequences of the present invention are included in the present invention.

Preferably, said derivative is a homologous variant which has conservative amino acid changes. By conservation amino acid changes we mean replacing an amino acid from one of the amino acid groups, namely hydrophobic, polar, acidic or basic, with an amino acid from within the same group. An examples of such a change is the replacement of valine by methionine and vice versa.

According to another aspect of the present invention there is provided a fusion polypeptide comprising at least one of the polypeptides of the present invention functionally linked to an appropriate non-*Heliothis* ecdysone receptor domain(s).

According to an especially preferred embodiment of the present invention the HEcR ligand binding domain of the present invention is fused to a DNA binding domain and a transactivation domain.

According to another embodiment of the present invention the DNA binding domain is fused to a ligand binding domain and a transactivation domain.

According to yet another embodiment of the present invention the transactivation domain is fused to a ligand binding domain and a DNA binding domain.

The present invention also provides recombinant DNA encoding for these fused polypeptides.

According to an especially preferred embodiment of the present invention there is provided recombinant nucleic acid comprising a DNA sequence encoding the HEcR ligand

binding domain functionally linked to DNA encoding the DNA binding domain and transactivation domain from a glucocorticoid receptor.

According to yet another aspect of the present invention there is provided recombinant nucleic acid comprising a DNA sequence comprising a reporter gene operably linked to a promoter sequence and a hormone response element which hormone response element is responsive to the DNA bonding domain encoded by the DNA of of the present invention.

According to another aspect of the present invention there is provided a construct transformed with nucleic acid, recombinant DNA, a polypeptide or a fusion polypeptide of the present invention. Such constructs include plasmids and phages suitable for transforming a cell of interest. Such constructs will be well known to those skilled in the art.

According to another aspect of the present invention there is provided a cell transformed with nucleic acid, recombinant DNA, a polypeptide, or a fusion polypeptide of the present invention.

Preferably the cell is a plant, fungus or mammalian cell.

For the avoidance of doubt fungus includes yeast.

The present invention therefore provides a gene switch which is operably linked to a foreign gene or a series of foreign genes whereby expression of said foreign gene or said series of foreign genes may be controlled by application of an effective exogenous inducer.

Analogs of ecdysone, such as Muristerone A, are found in plants and disrupt the development of insects. It is therefore proposed that the receptor of the present invention can be used be in plants transformed therewith as an insect control mechanism. The production of the insect-damaging product being controlled by an exogenous inducer. The insect-damagin g product can be ecdysone or another suitable protein.

The first non-steroidal ecdysteroid agonists, dibenzoyl hydrazines, typified by RH-5849 [1,2-dibenzoyl, 1-tert-butyl hydrazide], which is commercially available as an insecticide from Rohm and Haas, were described back in 1988. Another commercially available compound in this series is RH-5992 [tebufenozide, 3,5-dimethylbenzoic acid 1-1 (1,1-dimethylethyl)-2(4-ethylbenzoyl) hydrazide]. These compounds mimic 20-hydroxyecdysone (20E) in both *Manduca sexta* and *Drosophila melanogaster*. These compounds have the advantage that they have the potential to control insects using ecdysteroid agonists which are non-steroidal. Further Examples of such dibenzoyl hydrazines are given in US Patent No. 5,117,057 to Rohm and Haas, and Oikawa et al, Pestic Sci, 41, 139-148 (1994). However, it will be appreciated that any inducer of the gene switch of the present invention, whether steroidal or non-steroidal, and which is currently or becomes available, may be used.

The gene switch of the present invention, then, when linked to an exogenous or foreign gene and introduced into a plant by transformation, provides a means for the external regulation of expression of that foreign gene. The method employed for transformation of the plant cells is not especially germane to this invention and any method suitable for the target plant may be employed. Transgenic plants are obtained by regeneration from the transformed cells. Numerous transformation procedures are known from the literature such as agroinfection using *Agrobacterium tumefaciens* or its Ti plasmid, electroporation, microinjection or plants cells and protoplasts, microprojectile transformation, to mention but a few. Reference may be made to the literature for full details of the known methods.

Neither is the plant species into which the chemically inducible sequence is inserted particularly germane to the invention. Dicotyledonous and monocotyledonous plants can be transformed. This invention may be applied to any plant for which transformation techniques are, or become, available. The present invention can therefore be used to control gene expression in a variety of genetically modified plants, including field crops such as canola, sunflower, tobacco, sugarbeet, and cotton; cereals such as wheat, barley, rice, maize, and sorghum; fruit such as tomatoes, mangoes, peaches, apples, pears, strawberries, bananas and melons; and vegetables such as carrot, lettuce, cabbage and onion. The switch is also suitable for use in a variety of tissues, including roots, leaves, stems and reproductive tissues.

In a particularly preferred embodiment of the present invention, the gene switch of the present invention is used to control expression of genes which confer resistance herbicide resistance and/or insect tolerance to plants.

Recent advances in plant biotechnology have resulted in the generation of transgenic plants resistant to herbicide application, and transgenic plants resistant to insects. Herbicide tolerance has been achieved using a range of different transgenic strategies. One well documented example in the herbicide field is the use the bacterial xenobiotic detoxifying gene phosphinothricin acetyl transferase (PAT) from *Streptomyces hydroscopicus*. Mutated genes of plant origin, for example the altered target site gene encoding acetolactate synthase (ALS) from *Arabidopsis*, have been successfully utilised to generate transgenic plants resistant to herbicide application. The PAT and ALS genes have been expressed under the control of strong constitutive promoter. In the field of insecticides, the most common example to-date is the use of the Bt gene.

We propose a system where genes conferring herbicide and/or insect tolerance would be expressed in an inducible manner dependent upon application of a specific activating chemical. This approach has a number of benefits for the farmer, including the following:

1. Inducible control of herbicide and/or insect tolerance would alleviate any risk of yield penalties associated with high levels of constitutive expression of herbicide and/or insect resistance genes. This may be a particular problem as early stages of growth

where high levels of transgene product may directly interfere with normal development. Alternatively high levels of expression of herbicide and/or insect resistance genes may cause a metabolic drain for plant resources.

2. The expression of herbicide resistance genes in an inducible manner allows the herbicide in question to be used to control volunteers if the activating chemical is omitted during treatment.

3. The use of an inducible promoter to drive herbicide and/or insect resistance genes will reduce the risk of resistance becoming a major problem. If resistance genes were passed onto weed species from related crops, control could still be achieved with the herbicide in the absence of inducing chemical. This would particularly be relevant if the tolerance gene confirmed resistance to a total vegetative control herbicide which would be used (with no inducing chemical) prior to sowing the crop and potentially after the crop has been harvested. For example, it can be envisaged that herbicide resistance cereals, such as wheat, might outcross into the weed wild oats, thus conferring herbicide resistance to this already troublesome weed. A further example is that the inducible expression of herbicide resistance in sugar beet will reduce the risk of wild sugar beet becoming a problem. Similarly, in the field of insect control, insect resistance may well become a problem if the tolerance gene is constitutively expressed. The used of an inducible promoter will allow a greater range of insect resistance control mechanisms to be employed.

This strategy of inducible expression of herbicide resistance can be achieved with a pre-spray of chemical activator or in the case of slow acting herbicides, for example N-phosphonomethyl-glycine (commonly known as glyphosate), the chemical inducer can be added as a tank mix simultaneously with the herbicide. Similar strategies can be employed for insect control.

This strategy can be adopted for any resistance confering gene/corresponding herbicide combination, which is, or becomes, available. For example , the gene switch of the present invention can be used with:

1. Maize glutathione S-transferase (GST-27) gene (see our International Patent Publication No WO90/08826), which confers resistance to chloroacetanilide herbicides such as acetochlor, metolachlor and alachlor.

2. Phosphinotricin acetyl transferase (PAT), which confers resistance to the herbicide commonly known as glufosinate.

3. Acetolactate synthase gene mutants from maize (see our International Patent Publication No WO90/14000) and other genes, which confer resistance to sulphonyl urea and imadazolinones.

4. Genes which confer resistance to glyphosate. Such genes include the glyphosate oxidoreductase gene (GOX) (see International Patent Publication No. WO92/00377); genes which encode for 5-enolpyruvyl-3-phosphoshikimic acid synthase (EPSPS), including Class I and Class II EPSPS, genes which encode for mutant EPSPS, and genes which encode for EPSPS fusion peptides such as that comprised of a chloroplast transit peptide and EPSPS (see for example EP 218 571, EP 293 358, WO91/04323, WO92/04449 and WO92/06201); and genes which are involved in the expression of CPLyase.

Similarly, the strategy of inducible expression of insect resistance can be adopted for any tolerance confering gene which is, or becomes, available.

The gene switch of the present invention can also be used to controlled expression of foreign proteins in yeast and mammalian cells. Many heterologous proteins for many applications are produced by expression in genetically engineered bacteria, yeast cells and other eucaryotic cells such as mammalian cells.

As well as the obvious advantage in providing control over the expression of foreign genes in such cells, the switch of the present invention provides a further advantage in yeasts and mammalian cells where accumulation of large quantities of an heterologous protein can damage the cells, or where the heterologous protein is damaging such that expression for short periods of time is required in order to maintain the viability of the cells.

Such an inducible system also has applicability in gene therapy allowing the timing of expression of the therapeutic gene to be controlled. The present invention is therefore not only applicable to transformed mammalian cells but also to mammals *per se*.

A further advantage of the inducible system of the present invention in mammalian cells is that, because it is derived from a insect, there is less chance of it being effected by inducers which effect the natural mammalian steroid receptors.

In another aspect of the present invention the gene switch is used to switch on genes which produce potentially damaging or lethal proteins. Such a system can be employed in the treatment of cancer in which cells are transformed with genes which express proteins which are lethal to the cancer. The timing of the action of such proteins on the cancer cells can be controlled using the switch of the present invention.

The gene switch of the present invention can also be used to switch genes off as well as on. This is useful in disease models. In such a model the cell is allowed to grow before a specific gene(s) is switched off using the present invention. Such a model facilitates the study of the effect of a specific gene(s).

Again the method for producing such transgenic cells is not particularly germane to the present invention and any method suitable for the target cell may be used; such methods are known in the art, including cell specific transformation.

As previously mentioned, modulation of gene expression in the system appears in response to the binding of the HEcR to a specific control, or regulatory, DNA element. A schematic representation of the HEcR gene switch is shown in Figure 6. For ease of reference, the schematic representation only shows three main domains of the HEcR, namely the transactivation domain, DNA binding domain and the ligand binding domain. Binding of a ligand to the ligand binding domain enables the DNA binding domain to bind to the HRE resulting in expression (or indeed repression) of a target gene.

The gene switch of the present invention can therefore be seen as having two components. The first component comprising the HEcR and a second component comprising an appropriate HRE and the target gene. In practice, the switch may conveniently take the form of one or two sequences of DNA. At least part of the one sequence, or one sequence of the pair, encoding the HEcR protein. Alternatively, the nucleic acid encoding the HEcR can be replaced by the protein/ polypeptide itself.

Not only does the switch of the present invention have two components, but also one or more of the domains of the receptor can be varied producing a chimeric gene switch. The switch of the present invention is very flexible and different combinations can be used in order to vary the result/to optimise the system. The only requirement in such chimeric systems is that the DNA binding domain should bind to the hormone response element in order to produce the desired effect.

The glucocorticoid steroid receptor is well characterised and has been found to work well in plants. A further advantage of this receptor is that it functions as a homodimer. This means that there is no need to express a second protein such as the ultraspiracle in order to produce a functional receptor. The problem with the glucocorticoid steroid receptor is that ligands used to activate it are not compatible with agronomic practice.

In a preferred aspect of the present invention the receptor comprises glucocorticoid receptor DNA binding and transactivation domains with a *Heliothis* ligand binding domain according to the present invention. The response unit preferably comprising the glucocorticoid hormone response element and the desired effect gene. In the Examples, for convenience, this effect gene took the form of a reporter gene. However, in non-test or non-screen situations the gene will be the gene which produces the desired effect, for example produces the desired protein. This protein may be a natural or exogenous protein. It will be appreciated that this chimeric switch combines the best features of the glucocorticoid system, whilst overcoming the disadvantage of only being inducible by a steroid.

In another preferred embodiment, the *Heliothis* ligand binding domain is changed, and preferably replaced with a non-*Heliothis* ecdysone receptor ligand binding domain. For example, we have isolated suitable sequences from *Spodoptera exigua*.

Thus, according to another aspect of the present invention there is provided DNA having the sequence shown in Seq ID No. 6.

According to another aspect of the present invention there is provided DNA having part of the sequence shown in Seq ID No. 6, which encodes for the *Spodoptera* ecdysone ligand binding domain.

According to another aspect of the present invention there is provided DNA having part of the sequence shown in Seq ID No. 6, which encodes for the *Spodoptera* ecdysone hinge domain.

The present invention also provides the polypeptides coded for by the above DNA sequences of Seq ID No. 6.

A further advantage with such chimeric systems is that they allow you to choose the promoter which is used to drive the effector gene according to the desired end result. For example, placing the foreign gene under the control of a cell specific promoter can be particularly advantageous in circumstances where you wish to control not only the timing of expression, but also which cells expression occurs in. Such a double control can be particularly important in the areas of gene therapy and the use of cytotoxic proteins.

Changing the promoter also enables gene expression to be up- or down-regulated as desired.

Any convenient promoter can be used in the present invention, and many are known in the art.

Any convenient transactivation domain may also be used. The transactivation domain VP16 is a strong activator from Genentech Inc., and is commonly used when expressing glucocorticoid receptor in plants. Other transactivation domains derived for example from plants or yeast may be employed.

In a preferred embodiment of the present invention, the DNA binding domain is the glucocorticoid DNA binding domain. This domain is commonly a human glucocorticoid receptor DNA binding domain. However, the domain can be obtained from any other convenient source, for example, rats.

According to another aspect of the present invention there is provided a method of selecting compounds capable of being bound to an insect steroid receptor superfamily member comprising screening compounds for binding to a polypeptide or fusion polypeptide of the present invention, and selecting said compounds exhibiting said binding.

According to another aspect of the present invention there is provided a compound selected using the method of the present invention.

According to another aspect of the present invention there is provided an agricultural or pharmaceutical composition comprising the compound of the present invention.

According to yet another aspect of the present invention there is provided the use of the compound of the present invention as a pesticide, pharmaceutical and/or inducer of the switch. It will be appreciated that such inducers may well be useful as insecticides in themselves.

According to a further aspect of the present invention there is provided a method of producing a protein or peptide or polypeptide comprising introducing into a cell of the present invention, a compound which binds to the ligand binding domain in said cell.

Various preferred features and embodiments of the present invention will now be described by way of non-limiting example with reference to the accompanying examples and figures, in which figures:

Figure 1 (Sequence ID No. 1) shows the DNA sequence amplified from first strand cDNA made from mRNA isolated from *Heliothis virescens* Fourth instar larvae. The underlined sequences refer to the position of the degenerate oligonucleotides. At the 5' end the sequence matches that of the oligonucleotide while at the 3' end 12 nucleotides of the original oligonucleotide are observed;

Figure 2 (Sequence ID No. 2) shows the DNA sequence contained within the clone pSK19R isolated from a random primed cDNA *Heliothis virescens* library; Sequence is flanked by EcoRI sites;

Figure 3 (Sequence ID No. 3) shows the DNA sequence contained within the clone pSK16.1 isolated from a random primed cDNA *Heliothis virescens* library ;

Figure 4 (Sequence ID No. 4) DNA sequence of 5'RACE products (in bold) fused to sequence of clone pSK16.1. The ORF (open reading frame) giving rise to the *Heliothis virescens* ecdysone receptor protein sequence is shown under the corresponding DNA sequence;

Figure 5 (Sequence ID No. 5) shows the protein sequence alignment of the ecdysone receptors DmEcR (*Drosophila melanogaster*), CtEcR (*Chironomus tentans*), BmEcR (*Bombyx mori*), MsEcR (*Manduca sexta*), AaEcR (*Aedes aegipti*) and HvEcR (*Heliothis virescens*). "*" indicates conserved amino acid residue. "." indicates a conservative amino acid exchange;

Figure 6 shows a model of an embodiment of the glucocorticoid/*Heliothis* ecdysone chimeric receptor useable as a gene switch;

Figure 7 shows a plasmid map of the clone pcDNA319R. The three other mammalian expression vectors were constructed in the same way and look similar but for the size of the insert;

Figure 8 shows a plasmid map of the reporter construct used to analyse the activity of the *Heliothis virescens* ecdysone receptor;

Figure 9 is a graph which shows the effect of Muristerone A and RH5992 in reporter activity in HEK293 cells co-transfected with pcDNA3H3KHEcR alone (filled bars) or with αRXR (stripped bars);

Figure 10 shows a plasmid map of the Maize expression vector containing the Glucocorticoid receptor (HG1 or pMF6HG1PAT);

Figure 11 shows a plasmid map of the maize expression vector containing the chimeric glucocorticoid/*Drosophila* ecdysone receptor pMF6GREcRS;

Figure 12 shows a plasmid map of the maize expression vector containing the chimeric glucocorticoid/*Heliothis* ecdysone receptor pMF6GRHEcR;

Figure 13 shows a plasmid map of the plant reporter Plasmid containing the glucocorticoid response elements fused to the -60 S35CaMV promoter fused to GUS, p221.9GRE6;

Figure 14 shows a plasmid map of the plant reporter plasmid containing the glucocorticoid response elements fused to the -46 S35CaMV promoter fused to GUS, p221.10GRE6;

Figure 15 shows a graph showing the effect of Muristerone A and Dexamethasone in Maize AXB protoplasts transformed with pMF6HG1PAT (GR) and p221.9GRE6 (reporter);

Figure 16 shows a graph showing the effect of Muristerone A and Dexamethasone in Maize AXB protoplasts transformed with pMF6GREcRS (effector) and p221.9GRE6 (reporter);

Figure 17 shows a graph showing the effect of Muristerone A and Dexamethasone in Maize AXB protoplasts transformed with pMF6GRHEcR (effector) and p221.9GRE6 (reporter);

Figure 18 shows a graph showing the effect of RH5849 in Maize AXB protoplasts transformed with pMF6GREcRS (effector) and p221.9GRE6 (reporter);

Figure 19 shows a graph showing the effect of RH5992 in Maize AXB protoplasts transformed with pMF6GREcRS (effector) and p221.9GRE6 (reporter);

Figure 20 shows a graph showing the effect of RH5992 in Maize AXB protoplasts transformed with pMF6GRHEcR (effector) and p221.9GRE6 (reporter);

Figure 21 shows a graph which shows the dose response effect of RH5992 in Maize AXB protoplasts transformed with pMF6GRHEcR (effector) and p221.9GRE6 (reporter);

Figure 22 shows a plasmid map of the tobacco expression vector containing the chimeric glucocorticoid/ *Drosophila* ecdysone receptor, pMF7GREcRS;

Figure 23 shows a plasmid map of the tobacco expression vector containing the chimeric glucocorticoid/ *Heliothis* ecdysone receptor, pMF7GRHEcR;

Figure 24 shows a graph which shows the effect of RH5992 in Tobacco mesophyll protoplasts transformed with pMF6GRHEcR (Effector) and p221.9GRE6 (reporter);

Figure 25 shows a plasmid map of the mammalian expression vector containing the chimeric glucocorticoid/*Heliothis* ecdysone receptor, pcDNA3GRHEcR;

Figure 26 shows a plasmid map of the reporter plasmid pSWGRE4;

Figure 27 shows a graph which shows a RH5992 dose response curve of CHO cells transfected with pcDNA3GRHEcR and pSWGRE4;

Figure 28 shows a graph which shows the effect of Muristerone A and RH5992 on HEK293 cells co-transfected with pcDNA3GRHEcR and pSWGRE4;

Figure 29 shows a plasmid map of the binary vector ES1;

Figure 30 shows a plasmid map of the binary vector ES2;

Figure 31 shows a plasmid map of the binary vector ES3;

Figure 32 shows a plasmid map of the binary vector ES4;

Figure 33 shows a plasmid map of the effector construct TEV-B112 made to express the HEcR ligand binding domain in yeast;

Figure 34 shows a plasmid map of the effector construct TEV8 made to express the HEcR ligand binding domain in yeast;

Figure 35 shows a plasmid map of the effector construct TEVVP16-3 made to express the HEcR ligand binding domain in yeast;

Figure 36 shows a plasmid map of the mammalian expression vector containing the chimeric glucocorticoid VP16/*Heliothis* ecdsysone receptor, pcDNA3GRVP16HEcR;

Figure 37 shows a plasmid map of the maize expression vector containing the chimeric glucocorticoid VP16/*Heliothis* ecdsysone receptor, pMF6GRVP16HEcR;

Figure 38 shows a plasmid map of the maize expression vector containing the chimeric glucocorticoid VP16/*Heliothis* ecdsysone receptor, pMF7GRVP16HEcR;

Figure 39 shows a graph which shows the effect of RH5992 in Maize AXB protoplasts transformed with pMF6GRVP16HEcR (effector) and p221.9GRE6 (reporter);

Figure 40 (Sequence ID No. 6) shows the DNA sequence of the hinge and ligand binding domains of the *Spodoptera exigua* ecdysone receptor;

Figure 41 (Sequence ID No. 7) shows the protein sequence alignment of the *Heliothis* 19R and *Spodoptera* SEcR *Taq* clone hinge and ligand binding domains. "*" indicates conserved amino acid residue. "." indicates a conservative amino acid exchange;

Figure 42 shows a graph which shows the effect of RH5992 on Tobacco mesophyll protoplasts transformed with pMF7GRHEcR (effector) and either p221.9GRE6 (Horizontal strips) or p221.10GRE6 (vertical strips).

Example I - Cloning of the *Heliothis* Ecdysone Receptor

A. Probe generation

The rational behind the generation of the probe to isolate *Heliothis* homologues to the steroid/thyroid receptor superfamily members was based on comparing the sequences of developmentally regulated steroid/thyroid receptor superfamily members. The sequences available showed a highly conserved motif within the DNA binding domain of the RAR and THR (thyroid) receptors. The motifs were used to design degenerate oligonucleotides for PCR amplification of sequences derived from cDNA template produced from tissue expected to express developmentally regulated steroid/thyroid receptor superfamily members (ie. larval tissues).

The sense oligonucleotide is based on the peptide sequence CEGCKGFF which at the DNA level yields an oligonucletide with degeneracy of 32 as shown below :

```
ZnFA5'    5' TGC GAG GGI TGC AAG GAI TTC TT 3'
               T   A       T   A       T
```

The antisense oligonucleotide is based on the reverse complement nucleotide sequence derived from the peptide:

```
CQECRLKK
  S  R
```

for which four sets of degenerate oligos were made. Namely:

```
ZnFA3'  5' TTC TTI AGI CGG CAC TCT TGG CA 3'
             T           A   T   C   A

ZnFB3'  5' TTC TTI AAI CGG CAC TCT TGG CA 3'
             T           A   T   C   A

ZnFC3'  5' TTC TTI AGI CTG CAC TCT TGG CA 3'
             T           A   T   C   A

ZnFD3'  5' TTC TTI AAI CTG CAC TCT TGG CA 3'
             T           A   T   C   A
```

The PCR amplification was carried out using a randomly primed cDNA library made from mRNA isolated from 4th and 5th instar *Heliothis virescens* larvae. The amplification

was performed using 10^8 pfus (plaque forming units) in 50mM KCl. 20mM Tris HCl pH 8.4, 15mM MgCl2, 200mM dNTPs (an equimolar mixture of dCTP, dATP, dGTP and dTTP), 100ng of ZnFA5' and ZnF3' mixture. The conditions used in the reaction followed the hot start protocol whereby the reaction mixture was heated to 94°C for 5 minutes after which 1 U of Taq polymerase was added and the reaction allowed to continue for 35 cycles of 93°C for 50 seconds, 40°C for 1 minute and 73°C for 1 minute 30 seconds. The PCR products were fractionated on a 2%(w/v) agarose gel and the fragment migrating between 100 and 200bp markers was isolated and subcloned into the vector pCRII (Invitrogen). The sequence of the insert was determined using Sequenase (USB).

The resulting sequence was translated and a database search carried out. The search recovered sequences matching to the DNA binding domain of the *Drosophila* ecdysone receptor, retinoic acid receptor and the thyroid receptor. Thus, the sequence of the insert in this plasmid, designated pCRIIZnf, is a *Heliothis* ecdysone cognate sequence (Figure 1) and was used to screen a cDNA library in other to isolate the complete open reading frame.

B. Library screening

The randomly primed cDNA 4th/5th Instar *Heliothis virescens* library was plated and replicate filter made from the plates. The number of plaques plated was 500,000. The insert fragment of pCRIIZnf was reamplified and 50ng were end labelled using T4 Polynucleotide Kinase (as described in Sambrook et al 1990).

The filter were prehybridised using 0.25%(w/v) Marvel, 5 X SSPE and 0.1%(w/v) SDS at 42°C for 4 hours. The solution in the filters was ten replaced with fresh solution and the denatured probe added. The hybridisation was carried out overnight at 42°C after which the filter were washed in 6 X SSC + 0.1%(w/v) SDS at 42°C followed by another wash at 55°C. The filter were exposed to X-ray film (Kodak) for 48 hours before processing.

The developed film indicated the presence of one strong positive signal which was plaque purified and further characterised. The lambda ZAP II phage was in vivo excised (see Stratagene Manual) and the sequence determined of the resulting plasmid DNA. The clone known as pSK19R (or 19R) contained a 1.933kb cDNA fragment with an open reading frame of 467 amino acids (Figure 2). pSK19R was deposited with the NCIMB on 20 June 1995 and has been accorded the deposit No NCIMB 40743.

Further analysis of pSK19R revealed that a 340 bp EcoRI fragment mapping at the 5' end of pSK19R has strong and significant similarities to a *Drosophila* cDNA encoding glyceraldehyde-3-phosphate dehydrogenase. In order to isolate the correct 5'end sequence belonging to *Heliothis* , the random primed library was re-screened using a probe containing the 5'end of the pSK19R belonging to *Heliothis* ecdysone receptor. The probe was made by PCR using the sense oligonucleotide HecRH3C (5' aattaagcttccaccatgccgttaccaatgccaccgaca

3') and antisense oligonucleotide HecrNdeI (5' cttcaaccgacactcctgac 3'). The PCR was carried out as described by Hirst et al., 1992) where the amount of radioisotope used in the labelling was 50uCi of a ^{32}P-dCTP and the PCR was cycled for 1 minute at 94°C, 1minute at 60°C and 1 minute at 72°C for 19 cycles. The resulting 353bp radio labelled DNA fragment was denatured and added to prehybridised filters as described for the isolation of pSK19R. The library filters were made from 15 plates each containing 50000 pfus. The library filters were hybridised at 65°C and washed in 3XSSPE + 0.1%SDS at 65°C twice for 30 minutes each. The filters were further washed with 1XSSPE + 0.1%SDS for 30 minutes and exposed to X-ray film (Kodak) overnight. The film was developed and 16 putative positive plaques were picked. The plaques were re-plated and hybridised under the exact same conditions as the primary screen resulting in only one strong positive. The strong positive was consistently recognised by the probe and was plaque purified and *in vivo* excised. The resulting plasmid pSK16.1 was sequenced (Seq ID3) which revealed that the 5' end of the clone extended by 205 bp and at the 3' end by 653 bp and resulting in a DNA insert of 2.5 kb. Conceptual translation of the 205 bp yielded 73 amino acids with high similarity to the *Drosophila* , *Aedes aegipti*, *Manduca* and *Bombyx* sequences of the ecdsysone receptor B1 isoform. However, the whole of the 5' end sequence is not complete since a Methionine start site was not found with a stop codon in frame 5' of the methionine. In order to isolate the remainder of the 5' end coding sequences a 5'RACE protocol (Rapid Amplification of cDNA Ends) was carried out using the BRL-GIBCO 5'RACE Kit. Two types of cDNA were synthesised where the first one used a specific oligonucleotide :

16PCR2A 5' cagctccaggccgccgatctcg3'

and the second type used random hexamers (oligonucleotide containing 6 random nucleotides). Each cDNA was PCR amplified using the oligonucleotides anchor primer :

BRL-GIBCO 5' cuacuacuacuaggccacgcgtcgactagtacgggiigggiigggiig 3'

and 16PCR2A and cycled for 1 minute at 94°C, 1 minute at 60°C and 1 minute at 72°C for 35 cycles. The reaction conditions were 20mM Tris-HCl (pH8.4), 50mM KCl, 1.5mM $MgCl_2$, 400nM of each anchor and 16PCR2A primers, 200mM dNTPs (dATP,dCTP,dGTP and dTTP) and 0.02 U/ml *Taq* DNA polymerase. Dilutions of 1:50 of the first PCR reactions were made and 1ml was use in a second PCR with oligonucleotides UAP :

(Universal Amplification Primer 5' caucaucaucauggccacgcgtcgactagtac 3')

and 16RACE2 :

(5' acgtcacctcagacgagctctccattc 3').

The conditions and cycling were the same as those followed for the first PCR. Samples of each PCR were run and a Southern blot carried out which was probed with a 5' specific primer :

(16PCR1 5' cgctggtataacaacggaccattc 3').

This primer is specific for the 5' most sequence of pSK16.1 and was hybridised at 55°C using the standard hybridisation buffer. The filter was washed at 55°C 3 times in 3XSSPE + 0.1%SDS and exposed to X-ray film for up to 6 hours. The developed film revealed bands recognised by the oligonucleotide migrating at 100bp and 500bp (relative to the markers). A sample of the PCR reaction (4 in total) was cloned into the pCRII vector in the TA cloning kit (Invitrogen). Analysis of 15 clones from 4 independent PCRs yielded sequence upsteam of pSK16.1 (Figure 4).

Translation of the ORF results in a 575 amino acid protein with high similarity in the DNA and ligand binding domains when compared to the ecdysone receptor sequences of *Drosophila*, *Aedes aegypti*, *Chironomus tentans*, *Manduca sexta* and *Bombyx mori* (Figure 5). Interestingly, the N-terminal end of the *Heliothis* sequence has an in frame methinonine start which is 20 amino acids longer that that reported for *Drosophila*, *Aedes aegypti* and *Manduca sexta*. However, the extended N-terminal end in the *Heliothis* EcR does not have similarity to that of *Bombyx mori*. Finally, the C-terminal end of the different B1 isoform ecdysone receptor sequences diverge and do not have significant similarity.

C. Northern Blot Analysis

The sequence identified by screening the library is expected to be expressed in tissues undergoing developmental changes, thus mRNA from different developmental stages of H. virescens were was isolated and a northen blot produced. The mRNAs were isolated from eggs, 1st, 2nd, 3rd, 4th and 5th instar larvae, pupae and adults. The northern blot was hybridised with a NdeI/XhoI DNA fragment from pSK19R encompassing the 3'end of the DNA binding domain through to the end of the ligand binding domain. The hybridisation was carried out in 1%(w/v)Marvel, 5X SSPE, 0.1%(w/v) SDS at 65°C for 18 to 24 hours. The filters were washed in 3X SSPE + 0.1%(w/v) SDS and 1X SSPE + 0.1%(w/v) SDS at 65°C. The filter was blotted dry and exposed for one to seven days. The gene recognises two transcripts (6.0 and 6.5 kb) which appear to be expressed in all stages examined; however, the levels of expression differ in different stages. It should be noted that the same two transcripts are recognised by probes specific to the DNA binding domain and the ligand binding domain, indicating that the two transcripts arise from the same gene either by alternative splicing or alternative use of polyadenylation sites.

In summary, adult and 5th instar larvae have lower levels of expression while all other tissues have subtantial levels of expression.

Example II Expression of *Heliothis* ecdysone receptor in Mammalian cells

To demonstrate that the cDNA encodes a functional ecdysone receptor, effector constructs were generated containing the HEcR under the control of the CMV (cytomegalovirus) promoter, and the DNA expressed in mammalian cells.

Effector constructs

A first mammalian expression plasmid was constructed by placing a HindIII/NotI pSK19R fragment into the pcDNA3 HindIII/NotI vector resulting in pcDNA319R (Figure 7).

A second effector plasmid was constructed wherein the non-coding region of the cDNA 19R was deleted and a consensus Kozak sequence introduced. The mutagenesis was carried out by PCR amplifying a DNA fragment with the oligo HecRH3C :

5'aattaagcttccaccatgccgttaccaatgccaccgaca 3'

containing a unique HindIII restriction enzyme recognition site followed by the mammalian Kozak consensus sequence, and HecRNdeI :

5'cttcaaccgacactcctgac 3'.

The resulting 353bp PCR fragment was restriction enzyme digested with HindIII and NdeI, gel purified and ligated with 19R NdeI/NotI fragment into a pcDNA3 HindIII/NotI vector resulting in pcDNA3HecR.

A third effector construct was made with the 5' end sequences of pSK16.1 by PCR. The PCR approach involved PCR amplifying the 5' end sequences using a 5' oligonuclotide containing a HindIII restriction cloning site, the Kozak consensus sequence followed by nucleotide sequence encoding for a Methionine start and two Arginines to be added to the 5' end of the amplified fragment :

(16H3K 5' attaagcttgccgccatgcgccgacgctggtataacaacggaccattc 3'),

the 3' oligonucleotide used was HecrNdeI. The resulting fragment was restriction enzyme digested, gel purified and subcloned with an NdeI/NotI 19R fragment into pcDNA3 NdeI/NotI vector. The plasmid was named pcDNA3H3KHEcR.

A fourth effector construct was produced which contains the extended N-terminal end sequence obtained from the 5'RACE experiment. Thus, a PCR approach was followed to introduce the new 5' end sequences in addition to a consensus Kozak sequence and a HindIII unique cloning sequence. The sense oligonucleotide used was RACEH3K :

5' attaagcttgccgccatgtccctcggcgctcgtggatac 3',

while the antisense primer was the same as that used before (HecrNdeI). The cloning strategy was the same as used for the pcDNA3H3KHEcR to give rise to pcDNA3RACEH3KHEcR.

The PCR mutagenesis reactions were carried out in the same manner for all constructs. The PCR conditions used were 1 minute at 94°C, 1 minute at 60°C and 1 minute

at 72°C for 15 cycles. The reactions conditions were 50mM Tris-HCl (pH8.4), 25mM KCl, 200mM dNTPs (dATP, dCTP, dGTP and dTTP), 200nM of each oligonucleotide and 2.5U/Reaction of *Taq* DNA polymerase. For each construct at least 5 independant PCR reactions were carried out and several clones were sequenced to insure that at least one is mutation free.

Reporter construct

The reporter plasmid to be co-transfected with the expression vector contained 4 copies of the Hsp27 ecdysone response element (Riddihough and Pelham, 1987) fused to B-globin promoter and the B-Galactosidase gene. The tandem repeats of the ecdysone response element were synthesised as two complementary oligonucleotides which when annealed produced a double standed DNA molecule flanked by an SpeI site at the 5' end and a ClaI site at the 3' end :

Recr3A

5'ctagtagacaagggttcaatgcacttgtccaataagcttagacaagggttcaatgcacttgtccaatgaattcagacaagggttcaatgcacttgtccaatctgcagagacaagggttcaatgcacttgtccaatat 3'

Recr3B

5'cgatattggacaagtgcattgaacccttgtctctgcagattggacaagtgcattgaacccttgtctgaattcattggacaagtgcattgaacccttgtctaagcttattggacaagtgcattgaacccttgtcta 3'.

The resulting 135bp DNA fragment was ligated to the vector pSWBGAL SpeI/ClaI resulting in pSWREcR4 (Figure 8). The co-transfection of the two plasmid should result in B-galactosidase activity in the presence of ligand. The experiment relies upon the presence of RXR (a homologue of ultraspiracle) in mammalian cells for the formation of an active ecdysone receptor.

Mammalian transfection methods

Transfections of mammalian cell lines (CHO-K1 Chinese hamster ovary)- ATCC number CCL61 or cos-1 (Monkey cell line) were performed using either calcium phosphate precipitation (Gorman, Chapter 6 of "DNA cloning: a practical approach. Vol 2 D.M. Glover ed/.(1985) IRL Press, Oxford) or using LipofectAMINE (Gibco BRL Cat. No. 18324-012, following manufacturers instructions). Human Epithelial Kidney 293 cells were transfected using analogous methods.

Results - Native HEcR drives transient reporter gene expression in mammalian cells

Co-transfection of pcDNA3H3KHEcR (Effector) and reporter constructs into Human Epithelial Kidney 293 cells (HEK293) in the presence of either Muristerone A or RH5992 resulted in a 2-3 fold induction of reporter activity compared to the no chemical controls (Figure 9). The HEK293 cells were used since they are known to have constitutive levels of αRXR which have been demonstrated to be necessary for *Drosophila* EcR activation by Muristerone A (Yao., *et al.*, 1993). Moreover, to further investigate the need for RXR

interactions, a αRXR was co-transfected into HEK293 cells (along with the effector and reporter) resulting in a 9 fold induction of reporter activity compared to the untreated cells (Figure 9). The co-transfection of αRXR with reporter and effector increased by four fold the reporter activity compared to cells transfected with effector and reporter alone. Induction was observed both in the presence of either Muristerone A or RH5992. These data clearly demonstrate that the cDNA HEcR encodes a functional ecdysone receptor. Moreover, The ability of HEcR to complex with αRXR and bind Muristerone A or RH5992 provide evidence for the usage of the entire HEcR as a component of a mammalian gene switch. In particular, it offers the advantage of reducing uninduced expression of target gene since ecdysone receptor and response elements are not present in mammalian cells.

Example III - Chimeric constructs and ligand validation in Maize Protoplasts

In order to apply the ecdysone receptor as an inducible system it was deemed necesary to simplify the requirements of the system by avoiding the need of a heterodimer formation to obtain an active complex. The glucocorticoid receptor is known to form homodimers and chimeric constructs of the glucocorticoid receptor transactivating and DNA binding domains fused to the ecdysone receptor hinge and ligand binding domains have been shown to be active as homodimers in mammalian cells in the presence of Muristerone A (an ecdysone agonist)(Christopherson et al., 1992). However, the chimeric receptor is not responsive to 20-hydroxyecdysone (Christopherson et al., 1992).

The analysis of the activation of the glucocorticoid/*Heliothis* ecdysone chimeric receptor entailed the production of two other control effector constructs. The first one of the constructs contained the intact glucocorticoid receptor while the second one contained a glucocorticoid/*Drosophila* ecdysone chimeric receptor.

Effector constructs

(i) Glucocorticoid receptor Maize expression construct

The glucocorticoid receptor DNA for the Maize transient expression construct was produced via the polymerase chain reaction (PCR) of Human Fibrosarcoma cDNA (HT1080 cell line, ATCC#CCl121) library (Clontech)(see Hollenberg *et al.*, 1985). The PCR approach taken was to amplify the 2.7kb fragment encoding the glucocorticoid receptor in two segments. The first segment entails the N-terminal end up to and including the DNA binding domain while the second fragment begins with the hinge region (amino acid 500) thought to the end of the reading frame. Thus, the PCR primer for the N-terminal end segment was designed to contain an EcoRI site and the Kozak consensus sequence for translation initiation :

GREcoRI 5'attgaattccaccatggactccaaagaatcattaactc 3'.

The 3'end primer contains a XhoI site in frame with the reading frame at amino acid 500 of the published sequence :

GRXhoI 5' gagactcctgtagtggcctcgagcattccttttattttttc 3'.

The second fragment of the glucocorticoid receptor was produced with a 5' end oligonucleotide containing an XhoI site in frame with the open reading frame at the begining of the hinge region (amino acid 500) :

GRHinge 5' attctcgagattcagcaggccactacaggag 3'

while the 3' end oligonucleotide contained an EcoRI site 400 bp after the stop codon :

GRStop 5' attgaattcaatgctatcgtaactatacaggg 3'.

The glucocorticoid receptor PCR was carried out using Vent polymerase (Biolabs) under hot start conditions followed by 15 cycles of denaturing (94°C for 1 minute), annealing (66°C for 1 minute) and DNA synthesis (72°C for 3 minute). The template was produced by making first strand cDNA as described in the TA cloning kit (Invitrogen) after which the PCR was carried out in 10mM KCl, 10mM $(NH_4)_2SO_4$, 20mM TRIS-HCl pH 8.8, 2 mM $MgSO_4$, 0.1% (v/v) Triton X-100, 200 mM dNTPs, 100ng of each Primer and 2 U of Vent Polymerase. The PCR products was restriction enzyme digested with EcoRI and XhoI and subcloned into pBluescript SK (pSK) EcoRI. The resulting plasmid pSKHGI was sequenced and found to lack any mutations from the published sequences (apart from those introduced in the PCR primers) (Hollenberg et al., 1985).

The 2.7kb EcoRI fragment was subcloned into the vector pMF6PAT EcoRI resulting in pMF6HGIPAT (Figure 10).

(ii) Maize expression construct containing a Glucocorticoid/ *Drosophila* ecdysone chimeric receptor.

The glucocorticoid receptor portion of the chimeric receptor was isolated from pSKHGI by producing a 1.5kb BamHI/XhoI restriction fragment containing the N-terminal end up to and including the DNA binding domain.

The *Drosophila* ecdysone receptor portion was isolated through PCR of first stand cDNA prepared from *Drosophila* adult mRNA. The PCR was carried out using a 5' oligonucleotide containing a SalI site (ie. *Drosophila* ecdysone receptor contains a XhoI site at the end of the ligand binding domain) which starts at the begining of the hinge region : amino acid 330, Ecr8 attgtcgacaacggccggaatggctcgtcccggag 3'.

The 3' end oligonucleotide contains an BamHI site adjacent to the stop codon : EcRstop 5' tcgggctttgttaggatcctaagccgtggtcgaatgctccgacttaac 3'.

The PCR was carried out under the conditions described for the amplification of the Glucocorticoid receptor and yielded a 1.6 kb fragment. The fragment was introduced into

pSK SalI/BamHI and the sequence determined and compared to the published one (Koelle et al.,1991).

The maize transient expression plasmid was produced by introducing into pMF6 BamHI vector the 1.5kb BamHI/XhoI glucocorticoid receptor fragment and the 1.6kb SalI/BamHI *Drosophila* receptor portion to yield the chimeric plasmid pMF6GREcRS (Figure 9).

(iii) Construction of the Glucocorticoid/*Heliothis* ecdysone chimeric receptor Maize transient expression plasmid.

The Glucocorticoid receptor portion of the chimera was produced as describe in Example II(ii). The production of the *Heliothis* ecdysone receptor portion involves the introduction of a SalI recognition site at the DNA binding/hinge domain junction (amino acid 229). The addition of the SalI site :

Hecrsal 5'attgtcgacaaaggcccgagtgcgtggtgccggag 3'

was achieved via PCR mutagenesis making use of an unique AccI site 107bp downstream of the juction point (or 1007 bp relative to Seq 1D No 4):

Hecracc 5' tcacattgcatgatgggaggcatg 3'.

The PCR was carried out using *Taq* polymerase (2.5 U) in a reaction buffer containing 100ng of template DNA (pSK19R), 100ng of Hecrsal and Hecracc, 20 mM TRIS-HCl pH 8.4, 50mM KCl, 10mM $MgCl_2$, 200mM dNTPs. The reaction was carried out with an initial denaturation of 3 minutes followed by 15 cycles of denaturation (1 minute at 94°C), annealing (1 minute at 60°C) and DNA synthesis (1 minute at 72°C). The DNA was restriction enzyme digested and subcloned into pSK SalI/SacI with the 1.2kb AccI/SacI 3' end HecR fragment to yield pSK HeCRDEF (or containing the hinge and ligand binding domains of the *Heliothis* ecdysone receptor). The construction of the maize transient expression plasmid containing the Glucocorticoid/*Heliothis* ecdysone chimeric receptor involved the ligation of pMF6 EcoRI/SacI with the 1.5kb EcoRI/XhoI fragment of Glucocorticoid receptor N-terminal end and the 1.2 kb SalI/SacI fragment of pSk HEcRDEF to yield pMF6GRHEcR (Figure 10).

Reporter plasmids

Two reporter plasmids were made by inserting the into p221.9 or p221.10 BamHI/HindIII vectors two pairs or oligonucleotides containing six copies of the glucocorticoid response element (GRE). The two sets of oligonucleotides were designed with restriction enzyme recognition sites so as to ensure insertion of the two pairs in the right orientation. The first oligonucleotide pair GRE1A/B is 82 nucletides long and when annealed result in a DNA fragment flanked with a HindIII site at the 5' end and a SalI site at the 3' end :

GRE1A

5'agcttcgactgtacaggatgttctagctactcgagtagctagaacatcctgtacagtcgagtagctagaacatcctgtacag 3'

GRE1B

5'tcgactgtacaggatgttctagctactcgactgtacaggatgttctagctactcgagtcgctagaacatcctgta cagtcga 3'.

The second pair of oligonucleotides is flanked by a SalI site at the 5' end and a BamHI site at the the 3' end

GRE2A 5' tcgactagctagaacatcctgtacagtcgagtagctagaacatcctgt acagtcgagtagctagaacatcctgtacag 3'

GRE2B 5'gatcctgtacaggatgttctagctactcgactgtacaggatgttctagctactcgactgtacaggatgttctagctag 3'.

The resulting plasmids were named p221.9GRE6 (Figure 13) and p221.10GRE6 (Figure 14)(used in later Example). The difference between p221.9 and p221.10 plasmids is that p221.9 contains the -60 35SCaMV minimal promotor while p221.10 (p221.10GRE6) contains the -46 35SCaMV minimal promotor.

Method

Protoplasts were isolated from a maize suspension culture derived from BE70 x A188 embryogenic callus material, which was maintained by subculturing twice weekly in $MS0.5_{mod.}$ (MS medium supplemented with 3% sucrose, 690mg/l proline, 1g/l myo-inositol, 0.2g/l casein acid hydrolysate, 0.5mg/l 2,4-D, pH5.6). Cells from suspensions two days post subculture were digested in enzyme mixture (2.0% Cellulase RS, 0.2% Pectolyase Y23, 0.5M Mannitol, 5mM $CaCl_2 2H_2O$, 0.5% MES, pH5.6, ~660mmol/kg) using ~10ml/g cells, incubating at 25°C, dim light, rotating gently for ~2 hours. The digestion mixture was sieved sequentially through 250μm and 38μm sieves, and the filtrate centrifuged at 700rpm for 3.5 minutes, discarding the supernatant. The protoplasts were resuspended in wash buffer (0.358M KCl, 1.0mM NH_4NO_3, 5.0mM $CaCl_2 2H_2O$, 0.5mM KH_2PO_4, pH4.8, ~670mmol/kg) and pelleted as before. This washing step was repeated. The pellet was resuspended in wash buffer and the protoplasts were counted. Transformation was achieved using a Polyethylene glycol method based on Negrutiu et.al. Protoplasts were resuspended at 2×10^6/ml in MaMg medium (0.4M Mannitol, 15mM $MgCl_2$, 0.1% MES, pH5.6, ~450mmol/kg) aliquotting 0.5ml / treatment (i.e. 1×10^6 protoplasts / treatment). Samples were heat shocked at 45°C for 5 minutes then cooled to room temperature. 10μg each of p221.9GRE6 and pMF6HR1PAT (GR) (1mg/ml) / treatment were added and mixed in gently, followed by immediate addition of 0.5ml warm (~45°C) PEG solution (40% PEG 3,350MW in 0.4M Mannitol, 0.1M $Ca(NO_3)_2$, pH8.0), which was mixed in thoroughly but gently. Treatments were incubated at room temperature for 20-25 minutes, then 5ml 0.292M KCl (pH5.6, ~530mmol/kg) was added step-wise, 1ml at a time, with mixing. The treatments were incubated for a further 10-15 minutes prior to pelleting the protoplasts by centrifuging as before. Each protoplast treatment was resuspended in 1.5ml culture medium (MS medium, 2% sucrose, 2mg/l 2,4-D, 9% Mannitol, pH5.6, ~700mmol/kg) +/- 0.0001M dexamethasone (glucocorticoid). The samples were incubated in 3cm dishes at 25°C, dark, for 24-48 hours prior to harvesting. Fluorometric

assays for GUS activity were performed with the substrate 4-methylumbelliferyl-D-glucuronide using a Perkin-Elmer LS-35 fluorometer (Jefferson et al., 1987). Protein concentration of tissue homogenates were determined by the Bio-Rad protein assay (Bradford, 1976). The method was repeated for each effector construct.

Results

Reporter gene assay

A reporter gene construct (p221.9GRE6) was generated containing the GUS reporter gene under the control of a -60 CaMV 35S promoter with 6 copies of the glucocorticoid response element. To test this construct was functional in maize protoplasts a co-transformation assay was performed with the reporter construct p221.9GRE6 and the effector construct pMF6HR1PAT (GR) construct containing the entire glucorticoid receptor.

Figure 15 shows that Reporter p221.9GRE6 alone or reporter plus effector pMF6HR1PAT (GR) with no activating chemical gave no significant expression. When reporter plus effector were co-transformed into maize protoplasts in the presence of 0.0001M dexamethasone (glucocorticoid), a significant elevation of marker gene activity was observed (Figure 15). The response is specific to glucorticoid as the steroid Muristerone A does not lead to induced levels of expression. These studies clearly show the reporter gene construct p221.9GRE6 is capable of monitoring effector /ligand mediated gene expression.

Chimeric ecdysone effector constructs mediate inducible expression in maize transient protoplasts assays

A chimeric effector plasmid pMF6GREcRS was constructed, containing the ligand binding domain from the *Drosophila* ecdysone receptor and the DNA binding and transactivation domain from the glucorticoid receptor. To confirm the reporter gene construct p221.9GRE6 could respond to a chimeric ecdysone effector construct, a series of co-transformation into maize protoplasts was performed.

Figure 16 shows that reporter (p221.9GRE6) alone or reporter plus effector (pMF6GREcRS) with no activating chemical, gave no significant expression in maize protoplasts. When reporter plus effector were co-transformed into maize protoplasts in the presence of 100μM Muristerone A, a significant elevation of marker gene activity was observed. The response was specific to Muristerone A, as the steroid dexamethasone did not lead to induced levels of expression. These studies clearly showed the reporter gene construct p221.9GRE6 is capable of monitoring chimeric ecdysone effector /ligand mediated gene expression.

A second chimeric effector construct pMF6GRHEcR, was generated containing the ligand binding domain from *Heliothis* ecdysone receptor. When co-transformed into maize protoplasts with the reporter plasmid p221.9GRE6, no response to 100μM Muristerone or

100μM dexamethasone was observed (Figure 17). These data clearly show the *Drosophila* and *Heliothis* ligand binding domains exhibit different properties.

When the effector plasmid pMF6GREcRS, containing the ligand binding domain from *Drosophila*, was tested with the reporter p221.9GRE6 in presence of the non-steroidal ecdysone agonists RH5849 and RH5992 (mimic), no chemical induced reporter gene activity was observed (Figures 18 and 19).

When the effector plasmid pMF6GRHEcR, containing the ligand binding domain from *Heliothis*, was tested with the reporter p221.9GRE6 in presence of the non-steroidal ecdysone agonists RH5992 (mimic), significant chemical induced reporter gene activity was observed (Figure 20). These data demonstrate the ligand binding domain from *Heliothis* has different properties to the *Drosophila* receptor in that the former responded to the non-steroidal ecdysteroid agonist RH5992. Figure 21 demonstrates the effector plasmid pMF6GRHEcR confers RH5992 dependant inducibility on the reporter p221.9GRE6 in a dose responsive manner. Induction was observed in a range from 1μM-100μM RH5992.

Example IV - Testing of effector vectors in Tobacco protoplasts

The experiments carried out in the previous example demonstrated the specific effect of RH5992 (mimic) on pMF6GRHEcR in maize protoplasts. It is the aim in this example to show the generic application to plants of the glucocorticoid/*Heliothis* ecdysone chimeric receptor switch system. Tobacco shoot cultures cv. Samsun, were maintained on solidified MS medium + 3% sucrose in a controlled environment room (16 hour day / 8 hour night at 25°C, 55% R.H), were used as the source material for protoplasts. Leaves were sliced parallel to the mid-rib, discarding any large veins and the slices were placed in CPW13M 13% Mannitol, pH5.6, ~860mmol/kg) for ~1 hour to pre-plasmolyse the cells. This solution was replaced with enzyme mixture (0.2% Cellulase R10, 0.05% Macerozyme R10 in CPW9M (CPW13M but 9% Mannitol), pH5.6, ~600mmol/kg) and incubated in the dark at 25°C overnight (~16 hours). Following digestion, the tissue was teased apart with forceps and any large undigested pieces were discarded. The enzyme mixture was passed through a 75μm sieve and the filtrate was centrifuged at 600rpm for 3.5 minutes, discarding the supernatant. The pellet was resuspended in 0.6M sucrose solution and centrifuged at 600rpm for 10 minutes. The floating layer of protoplasts was removed using a pasteur pipette and diluted with CPW9M (pH5.6, ~560mmol/kg). The protoplasts were again pelleted by centrifuging at 600rpm for 3.5 minutes, resuspended in CPW9M and counted. A modified version of the PEG-mediated transformation above was carried out. Protoplasts were resuspended at $2x10^6$/ml in MaMg medium and aliquotted using 200μl / treatment (i.e. $4x10^5$ protoplasts / treatment). 20μg each of pMF6GRHEcRS and p221.9GRE6 DNA (1mg/ml) were added

followed by 200µl PEG solution and the solutions gently mixed. The protoplasts were left to incubate at room temperature for 10 minutes before addition of 5ml MSP19M medium (MS medium, 3% sucrose, 9% Mannitol, 2mg/l NAA, 0.5mg/l BAP, pH5.6, ~700mmol/kg) +/- 10 µM RH5992. Following gentle mixing, the protoplasts were cultured in their tubes, lying horizontally at 25°C, light. The protoplasts were harvested for the GUS assay after ~24 hours.

Effector construct

(i) Construction of a Dicotyledonous expression vector

The vector produced is a derivative of pMF6. pMF6GREcRS was restriction enzyme digested with PstI to produce 3 fragments namely, 3.4(Adh Intronless pMF6), 3.2(GREcRS) and 0.5(Adh intron I) kb). Isolation and religation of the 3.4 and 3.2 kb fragments resulted in pMF7GREcRS (Figure 22). pMF7GREcRS was restriction enzyme digested with EcoRI/SacI resulting in the 3.4kb pMF7 EcoRI/SacI vector which when isolated and purified was ligated to a 1.5 kb EcoRI/XhoI N-terminal end of the glucocorticoid receptor and the 1.2 kb SalI/SacI *Heliothis* ecdysone C-terminal end sequences to produce pMF7GRHEcR (Figure 23).

Reporter plasmid

The reporter plasmids constructed for the maize transient experiments were the same as those used without alteration in the tobacco leaf protoplast transient expression experiments.

Results - Chimeric ecdysone effector constructs mediate inducible expression in tobacco transient protoplast assays

Experiments were performed to demonstrate that the effector plasmid pMF6GRHEcR can confer chemical dependant inducible expression on the reporter p221.9GRE6 in tobacco mesophyll protoplasts.

Figure 24 shows that reporter (p221.9GRE6) alone or reporter plus effector (pMF7GRHEcR) with no activating chemical, gave no significant expression in tobacco protoplasts. When reporter plus effector were co-transformed into tobacco protoplasts in the presence of 10µM RH5992, a significant elevation of marker gene activity was observed. These data show a chimeric ecdysone effector construct, containing the *Heliothis* ligand binding domain can confer non-steroidal ecdysteroid dependant expression on reporter gene constructs in both monocotyledonous and dicotyledonous species.

Example V - Chimeric activity in Mammalian cells

Effector constructs

(i) Construction of Glucocorticoid/*Heliothis* ecdysone chimeric receptor.

The mammalian expression vector used in this experiment was pcDNA3 (Invitrogen). The GRHEcR 2.7kb BamHI DNA fragment (isolated from pMF6GRHEcR) was introduced into the pcDNA3 BamHI vector. The recombinants were oriented by restriction enzyme mapping. The DNA sequence of the junctions was determined to ensure correct orientation and insertion (pcDNA3GRHEcR, Figure 25).

Reporter construct

The reporter plasmid for mammalian cell system was produced by taking pSWBGAL plasmid and replacing the CRESW SpeI/ClaI fragment for a synthetic 105 bp DNA fragment containing 4 copies of the glucocorticoid response element (GRE) and flanked by SpeI at the 5' end and Af1II at the 3' end.

The oligonucleotides were synthesised using the sequences :

GREspeI

5'ctagttgtacaggatgttctagctactcgagtagctagaacatcctgtacagtcgagtagctagaacatcctgtacagtcgagtagct agaacatcctgtacac 3'

GREafl2

5'ttaagtgtacaggatgttctagctactcgactgtacaggatgttctagctactcgactgtacaggatgttctagctactcgagtagcta gaacatcctgtacaa 3'.

The two oligonucleotides were purified annealed and ligated to pSWBGAL SpeI/AflII to produce pSWGRE4 (Figure 26).

Results - Chimeric HEcR drives transient reporter gene expression in mammalian cells

No expression was detected when a reporter gene construct pSWGRE4, comprising of a minimal β-globin promoter containing four copies of the glucocorticoid response element, fused to a β-galactosidase reporter gene, was introduced into CHO cells. Similarly, no expression was detected when pSWGRE4 and an effector plasmid pCDNA3GRHEcR, containing the transactivation and DNA binding domain from the glucocorticoid receptor and the ligand binding domain from the *Heliothis* ecdysone receptor, under the control of the CMV promoter were co-transformed into CHO-K1 or HEK293 cells. When co-transformed CHO (Figure 27) and HEK293 cells (Figure 28) were incubated in the presence of the non-steroidal ecdysone agonists RH5992 (mimic), significant chemical induced reporter gene activity was observed. Equally, induction of reporter activity was observed when HEK293 cells transfected with pcDNA3GRHEcR and reporter were treated with Muristerone A (Figure 28).

Example VI - Screening system allows new chemical activators and modified ligand binding domains to be tested in Mammalian cells

The basis of a screening system are in place after the demonstration that the chimeric receptor was activated in the presence of RH5992. A screen was carried out using CHO cells transiently transfected with both pSWGRE4 (reporter) and pcDNA3GRHEcR (effector) constructs. In the first instance 20 derivatives compounds of RH5992 were screened. It was observed that 7 out of the 20 compounds gave an increased reporter gene activity compared to untreated cells. A second screen was carried out in which 1000 randomly selected compounds were applied to transiently transfected CHO cells. Two compounds were found to activate reporter gene activity above that from the untreated controls. The second screen suggest that this cell based assay is a robust and rapid way to screen a small library of compounds, where a thousand compounds can be put through per week.

Example V - Stably transformed Tobacco plants

<u>Stable Tobacco vectors</u>

The components of the stable Tobacco vectors were put together in pBluescript prior to transfer into the binary vector. The production of stable transformed plants entails the production of a vector in which both components of the switch system (ie. effector and reporter) are placed in the same construct to then introduce into plants.

The methodology described below was used to produce four different stable Tobacco vectors. The method involves three steps:

1. pBluescript SK HindIII/EcoRI vector was ligated to either GRE6-4635SCaMVGUSNOS HindIII/EcoRI (from p221.10GRE6) or GRE6-6035SCaMVGUSNOS HindIII/EcoRI (from p221.9GRE6) resulting in plasmid pSK-46 and pSK-60.

2. This step involves the addition of the chimeric receptor (35SGRHEcRNOS or 35SGRVP16HEcRNOS) to pSK-60 or pSK-46. Thus a pSK-60 (or pSK-46) XbaI vector was ligated with either the 3.4kb 35SGRHEcRNOS XbaI or the 3.0kb 35SGRVP16HEcRNOS XbaI DNA fragment to produce pSKES1 (pSKGRE6-6035SCaMVGUSNOS-35SGRHEcRNOS), pSKES2 (pSKGRE6-4635SCaMVGUSNOS-35SGRHEcRNOS), pSKES3 (pSKGRE6-6035SCaMVGUSNOS-35SGRVP16HEcRNOS) and pSKES4 (pSKGRE6-4635SCaMVGUSNOS-35SGRVP16HEcRNOS).

3. Transfer from pBluescript based vectors to binary vectors: The transfer of the ES1 (Figure 29) ES2 (Figure 30), ES3 (Figure 31) or ES4 (Figure 32) DNA fragments into the binary vector JR1 involves five steps:

(i) Restriction enzyme digestion of pSKES1 (ES2, ES3,and ES4) with ApaI and NotI to liberate the insert from the vector pBluescript.

(ii) The two DNA fragments were BamHI methylated for 2 hours at 37°C in TRIS-HCl, MgCl, 80uM SAM (S-adenosylmethionine) and 20 U of BamHI methylase.

(iii) Ligate a ApaI/NotI linker onto the fragment. The linker was designed to have an internal BamHI site :

ApaBNot1 5' cattggatccttagc 3' and

ApaBNot2 5'ggccgctaaggatccaatgggcc 3'.

(iv) Restriction enzyme digest the protected and linkered fragment with BamHI and fractionate the products on a 1%(w/v) Agarose gel. The protected DNA fragment (5.5kb) was cut out of the gel and purified.

(v) A ligation of JRI BamHI vector with the protected band was carried out to produce JRIESI (JRIES2, JRIES3 or JRIES4). The DNA of the recombinant was characterised by restriction mapping and the sequence of the junctions determined.

The plant transformation construct pES1, containing a chimeric ecdysone receptor and a reporter gene cassette, was transferred into Agrobacterium tumefaciens LBA4404 using the freeze/thaw method described by Holsters et al. (1978). Tobacco (Nicotiana tabacum cv Samsun) transformants were produced by the leaf disc method (Bevan, 1984). Shoots were regenerated on medium containing 100mg/l kanamycin. After rooting, plantlets were transferred to the glasshouse and grown under 16 hour light/ 8 hour dark conditions.

<u>Results - Chimeric ecdysone effector constructs mediate inducible expression in stably tobacco plants</u>

Transgenic tobacco plants were treated in cell culture by adding 100μM RH5992 to MS media. In addition seedlings were grown hydroponically in the presence or absence of RH5992. In further experiments 5mM RH5992 was applied in a foliar application to 8 week old glasshouse grown tobacco plants. In the three methods described uninduced levels of GUS activity were comparable to a wild type control, while RH5992 levels were significantly elevated.

Ecdysone switch modulation and optimisation

Example VIII - Yeast indicator strains for primary screen of chemical libraries

A set of yeast indicator strains was produced to use as a primary screen to find chemicals which may be used in the gene switch. The properties of the desired chemicals should include high affinity resulting in high activation but with different physico-chemical characteristics so as to increase the scope of application of the technology. Moreover, the production of this strain also demonstrates the generic features of this switch system.

Effector vector

A base vector for yeast YCp15Gal-TEV-112 was generated containing:
Backbone - a modified version of pRS315 (Sikorski and Hieter (1989) Genetics 122, 19-27)- a shuttle vector with the LEU2 selectable marker for use in yeast;
ADH1 promoter (BamHI- Hind III fragment) and ADH1 terminator (Not I- Bam HI fragment) from pADNS (Colicelli et al PNAS 86, 3599-3603);
DNA binding domain of GAL4 (amino acids 1-147; GAL4 sequence is Laughon and Gesteland 91984) Mol. Cell Biol. 4, 260-267) from pSG424 (Sadowski and Ptashne (1989) Nuc. Acids Res. 17, 7539);
Activation domain - an acidic activation region corresponding to amino acids 1-107 of activation region B112 obtained from plasmid pB112 (Ruden et al (1991) Nature 350, 250-252).

The plasmid contains unique Eco RI, Nco I and Xba I sites between the DNA binding domain and activation domains.

Into this vector a PCR DNA fragment of the *Heliothis* ecdysone receptor containing the hinge, ligand binding domains and the C-terminal end was inserted. The 5' oligonucleotide is flanked by an NcoI restriction recognition site and begins at amino acid 259 :
HecrNcoI 5' aattccatggtacgacgacagtagacgatcac 3'.

The 3' oligonucleotide is flanked by an XbaI site and encodes for up to amino acid 571:
HecRXbaI 5' ctgaggtctagagacggtggcgggcggcc 3'.

The PCR was carried out using vent polymerase with the conditions described in Example IA. The fragment was restriction enzyme digested with NcoI and XbaI purified and ligated into YCp15GALTEV112 NcoI/XbaI vector to produce YGALHeCRB112 or TEV-B112 (Figure 34). In order to reduce constitutive activity of the YGALHeCRB112 plasmid a YGALHeCR plasmid was produced in which the B112 activator was deleted by restriction enzyme digesting YGALHeCRB112 with XbaI/SpeI followed by ligation of the resulting

vector (ie. SpeI and XbaI sites when digested produce compatible ends)(TEV-8, Figure 33). An effector plasmid was constructed whereby the B112 transactivating domain was excised from YGalHecRB112 with XbaI and replaced with the VP16 transactivation domain DNA fragment (encoding amino acids 411 and 490 including the stop codon). The resulting vector was named YGalHecRVP16 or TEVVP16-3 (Figure 35).

Reporter construction for yeast

The S. cerevisiae strain GGY1::171 (Gill and Ptashne (1987) Cell 51, 121-126), YT6::171 (Himmelfarb et al (1990) Cell 63, 1299-1309) both contain reporter plasmids consisting of the GAL4-responsive GAL1 promoter driving the E. coli B-galactosidase gene. These plasmids are integrated at the URA3 locus. The reporter strain YT6::185 contains the reporter plasmid pJP185 (two synthetic GAL4 sites driving the B-galactosidase gene) integrated at the URA3 locus of YT6 (Himmelfarb et al). (Note- the parental strains YT6 and GGY1 have mutations in the GAL4 and GAL80 genes, so the reporter genes are inactive in the absence of any plasmids expressing GAL4 fusions).

Yeast assay

Standard transformation protocols (Lithium acetate procedure) and selection of colonies by growth of cells on selective media (leucine minus medium in the case of the YCp15Gal-TEV-112 plasmid)- as described in Guthrie and Fink)1991) Guide to Yeast Genetics and Molecular Biology: Methods in Enzymology Vol. 194 Academic Press) and the reporter gene assay is a modification of that described in Ausabel et al (1993) Current Protocols in Molecular Biology (Wiley) Chapter 13).

Results - Automated screening system allows new chemical activators and modified ligand binding domains to be tested in yeast

An effector vector pYGALHEcRB112 has been generated containing a GAL4 DNA binding domain, a B112 activation domain and the ligand binding region from *Heliothis virescens*. In combination with a GAL reporter vector, pYGALHEcRB112 form the basis of a rapid, high throughput assay which is cheap to run. This cell-based assay in yeast (*Saccharomyces cerevisiae*) will be used to screen for novel non-steroidal ecdysone agonists which may of commercial interest as novel insecticides or potent activators of the ecdysone gene switch system. The demonstration of an efficient system to control gene expression in a chemical dependant manner, forms the basis of an inducible system for peptide production in yeast.

The yeast screening system forms the basis of a screen for enhanced ligand binding using the lac Z reporter gene vector to quantitatively assay the contribution of mutation in the ligand binding domain. Alternatively, enhanced ligand binding capabilities or with a selection cassette where the lac Z reporter is replaced with a selectable marker such as uracil (URA 3), tryptophan (Trp1) or leucine (Leu2), and histidine (His). Constructs based on

pYGALHEcRB112 with alterations in the ligand binding domain are grown under selection conditions which impair growth of yeast containing the wild type ligand binding domain. Those surviving in the presence of inducer are retested and then sequenced to identify the mutation conferring resistance.

Example IX - Optimisation of chimeric receptor using a strong transactivator

Construction of mammalian expression plasmid with chimeric receptor containing Herpex Simplex VP16 protein sequences.

The construction of this chimeric receptor is based on replacing the sequences encoding for the glucocorticoid receptor transactivating domain with those belonging to the VP16 protein of Herpex simplex. Thus PCR was used to generate three fragments all to be assembled to produce the chimeric receptor. The PCRs were carried out as described in Example II, iii. The first fragment includes the Kozak sequences and methionine start site of the glucocorticoid receptor to amino acid 152 of the glucocorticoid receptor. The oligonucleotides used for the generation of this fragment included an EcoRI site at the 5' end:

GR1A 5' atatgaattccaccatggactccaaagaatc 3'

and at the 3' end a NheI restriction enzyme recognition site :

GR1B 5' atatgctagctgtgggggcagcagacacagcagtgg 3'.

The second fragment also belongs to the glucocorticoid receptor and begins with a NheI site in frame with amino acid 406 :

GR2A 5'atatgctagctccagctcctcaacagcaacaac 3'

and ends with a XhoI site at amino acid 500 :

GR2B 5'atatctcgagcaattccttttattttttc 3'.

The two fragments were introduced into pSKEcoRI/SacI in a ligation containing GR1A/B EcoRI/NheI, GR2A/B NheI/XhoI and HEcR SalI/SacI (from pSKHEcRDEF) to yield pSKGRDHEcR. The GR sequences and junctions of the ligation were found to be mutation free.

The third fragment to be amplified was a sequence between amino acid 411 to 490 of the herpes simplex VP16 protein. The amplified fragment was flanked with SpeI recognition sites. SpeI produces compatible ends to those of NheI sites. The oligonucleotides used :

VP16C 5' attactagttctgcggcccccccgaccgat 3' and

VP16E 5' aattactagtcccaccgtactcgtcaattcc 3'

produced a 180 bp fragment which was restriction enzyme digested with SpeI and introduced into pSKGRΔHEcR NheI vector to produce pSKGRVP16HEcR. The DNA from the latter was sequenced and and found to be mutation free, the junctions were also shown to be in frame with those of the glucocorticoid receptor.

The 2.2 kb EcoRV/NotI GRVP16HEcR fragment was introduced into a pcDNA3 EcoRV/NotI vector resulting in pcDNA3GRVP16HEcR (Figure 36).

Construction of plant transient expression effector plasmids containing the chimeric receptor with VP16 sequences

The same procedure was carried out to clone the GRVP16HeCR DNA fragment into tobacco(pMF7b) and maize(pMF6) expression vectors. A 2.2kb BamHI DNA fragment was isolated from pcDNA3GRVP16HeCR and ligated in to the pMF6 BamHI (or pMF7b BamHI) vector to produce pMF6GRVP16HeCR (Figure 37) (or pMF7GRVP16HeCR) (Figure 38).

Results - Addition of strong activation domains enhance ecdysone switch system

The VP16 transactivation domain from herpes simplex virus has been added to a maize protoplast vector pMF6GRHEcR to generate the vector pMF6GRVP16HEcR. When co-transformed into maize protoplasts with the reporter construct p221.9GRE6, in the presence of 100μM RH5992, enhanced levels of expression were seen over pMF6GRHEcR. Figure 39, shows that RH5992 is able to induce GUS levels comparable to those observed with the positive control (p35SCaMVGUS), moreover, a dose response effect is observable.

VP16 enhanced vectors (pES3 and pES4) have been generated for stable transformation of tobacco. Following transformation transgenic progeny containing pES3 and pES4, gave elevated GUS levels following treatment with RH5992, relative to comparable transgenic plants containing the non-VP16 enhanced vectors pES1 and pES2.

An enhanced mammalian vector pcDNA3GRVP16HEcR was prepared for transient transfection of mammalian cell lines. Elevated reporter gene activities were obtained relative to the effector construct (pCDNA3GRHEcR) without the VP16 addition.

"Acidic" activation domains are apparently "universal" activators in eukaryotes (Ptashne (1988) Nature 335 683-689). Other suitable acidic activation domains which have been used in fusions are the activator regions of GAL4 itself (region I and region II; Ma and Ptashne (Cell (1987) 48, 847-853), the yeast activator GCN4 (Hope and Struhl (1986) Cell 46, 885-894) and the herpes simplex virus VP16 protein (Triezenberg et al (1988) Genes Dev. 2, 718-729 and 730-742).

Other acidic and non-acidic transcriptional enhancer sequences for example from plant fungal and mammalian species can be added to the chimeric ecdysone receptor to enhance induced levels of gene expression.

Chimeric or synthetic activation domains can be generated to enhance induced levels of gene expression.

Example X - Optimisation by replacement of *Heliothis* ligand binding domain in chimeric effector for that of an ecdysone ligand binding domain of another species

Mutagenesis of the ecdysone ligand binding domain results in the increased sensitivity of the chimeric receptor for activating chemical. This can be achieved by deletions in the ligand binding domain, use of error prone PCR (Caldwell et al., PCR Meth. Applic 2, 28-33 1992), and in vitro DNA shuffling PCR (Stemmer, Nature 370, 389-391 1994). To enhance the efficacy of the listed techniques we have developed a screening system for enhanced levels of induced expression (see below).

An alternative strategy to the mutation of a known ligand binding domain is to identify insect species which are particularly sensitive to ecdysteroid agonists. For example *Spodoptera exigua* is particularly sensitive to RH 5992. To investigate the role of the ecdysone receptor ligand binding domain in increased sensitivity to RH5992 we have isolated corresponding DNA sequences from of *S. exigua* (Figure 40, Sequence ID No. 6). Figure 41, Sequence ID No. 7 shows a protein alignment of the hinge and ligand binding domains of the *Heliothis virescens* and *Spodoptera exigua* ecdysone receptors. The protein sequence between the two species is well conserved.

Results - Manipulation of the ligand binding domain leads to enhanced induced expression

Isolation of an ecdysone ligand binding domain from another lepidopteran species was carried out by using degenerate oligonucleotides and PCR of first strand cDNA (Perkin Elmer, cDNA synthesis Kit) of the chosen species. The degenerate oligonucleotides at the 5' end were HingxhoA and B and at the 3' end ligandxA/B

```
HingxhoA  5' attgctcgagaaagiccigagtgcgtigticc 3'
                                  a  t
HingxhoB  5' attgctcgagaacgiccigagtgtgtigticc 3'
                                  a  c

LigandxA  5' ttactcgagiacgtcccaiatctcttciaggaa 3'
                        a        t  c     a

ligandxB  5' ttactcgagiacgtcccaiatctcctciaagaa 3'
                        a        t   t    a
```

RNA was extracted from 4th instar larvae of *Spodoptera exigua* since *Spodoptera exigua* appears to be more sensitive to RH5992 than *Heliothis* (Smagghe and Degheele,

1994). The first strand cDNA was used in PCR reactions under the following conditions 20mM Tris-HCL (pH8.4), 50mM KCl, 1.5mM $MgCl_2$, 200mM dNTPs (dATP,dCTP,dGTP and dTTP) and 0.02 U/ml *Taq* DNA polymerase and in the presence of 1ug of each Hinge (5' 3') and Ligand (5'3') oligonucleotides. The PCR cycling conditions were 94°C for 1 minute, 60°C for 2 minutes and 72°C for 1 minute and 35 cycles were carried out. A sample of the completed reaction was fractionated in a 1% agarose (w/v) 1 x TBE gel, and the resulting 900 bp fragment was subcloned into pCRII vector (Invitrogen). The resulting clone (pSKSEcR 1-10) were further characterised and sequenced.

Example X - Manipulation of reporter gene promoter regions can modulate chemical induced expression

The context of the effector response element in the reporter gene promoter can be used to modulate the basal and induced levels of gene expression. Six copies of the glucorticoid response element were fused to 46 bp or 60 bp of the CaMV 35S promoter sequence. When used with the effector construct pMF7GRHEcRS the reporter gene construct containing 46 bp of the CaMV 35S promoter gave reduced basal and induced levels of GUS expression relative to the 60 bp reporter construct (Figure 42).

Constructs for plant transformation (pES1 and ES2) have been generated to demonstrate the size of minimal promoter can be used to modulate the basal and induced levels of gene expression in plants.

The number and spacing of response elements in the reporter gene promoter can be adjusted to enhance induced levels of trans-gene expression.

The utility of a two component system (effector and reporter gene cassettes) allows the spatial control of induced expression. Trans-gene expression can be regulated in an tissue specific, organ specific or developmentally controlled manner. This can be achieved by driving the effector construct from a spatially or temporally regulated promoter.

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: ZENECA LIMITED
(B) STREET: 15 STANHOPE GATE
(C) CITY: LONDON
(E) COUNTRY: UK
(F) POSTAL CODE (ZIP): W1Y 6LN

(ii) TITLE OF INVENTION: A GENE SWITCH

(iii) NUMBER OF SEQUENCES: 7

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(vi) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: GB 9510759.5
(B) FILING DATE: 26-MAY-1995

(vi) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: GB 9513882.3
(B) FILING DATE: 07-JUL-1995

(vi) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: GB 9517316.7
(B) FILING DATE: 24-AUG-1995

(vi) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: GB 9605656.9
(B) FILING DATE: 18-MAR-1996

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 116 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA to mRNA

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Heliothis virescens

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

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(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1934 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: circular

(ii) MOLECULE TYPE: cDNA

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Heliothis virescens

(vii) IMMEDIATE SOURCE:
(B) CLONE: pSK19R

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

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(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2464 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: circular

(ii) MOLECULE TYPE: cDNA

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Heliothis virescens

(vii) IMMEDIATE SOURCE:
(B) CLONE: pSK16.1

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

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(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2745 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION:225..1955
(D) OTHER INFORMATION:/codon_start= 225
/product= "Heliothis ecdysone receptor"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

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(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 575 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

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            20   25   30

Leu Glu Glu Ser Ser Ser Glu Val Thr Ser Ser Ser Ala Leu Gly Leu
        35   40   45

Pro Pro Ala Met Val Met Ser Pro Glu Ser Leu Ala Ser Pro Glu Ile
    50   55   60

Gly Gly Leu Glu Leu Trp Gly Tyr Asp Asp Gly Ile Thr Tyr Ser Met
65   70   75   80

Ala Gln Ser Leu Gly Thr Cys Thr Met Glu Gln Gln Gln Pro Gln Pro
                85   90   95

Gln Gln Gln Pro Gln Gln Thr Gln Pro Leu Pro Ser Met Pro Leu Pro
            100   105   110

Met Pro Pro Thr Thr Pro Lys Ser Glu Asn Glu Ser Met Ser Ser Gly
        115   120   125

Arg Glu Glu Leu Ser Pro Ala Ser Ser Val Asn Gly Cys Ser Thr Asp
    130   135   140

Gly Glu Ala Arg Arg Gln Lys Lys Gly Pro Ala Pro Arg Gln Gln Glu
145   150   155   160

Glu Leu Cys Leu Val Cys Gly Asp Arg Ala Ser Gly Tyr His Tyr Asn
                165   170   175

Ala Leu Thr Cys Glu Gly Cys Lys Gly Phe Phe Arg Arg Ser Val Thr
            180   185   190

Lys Asn Ala Val Tyr Ile Cys Lys Phe Gly His Ala Cys Glu Met Asp
        195   200   205

Ile Tyr Met Arg Arg Lys Cys Gln Glu Cys Arg Leu Lys Lys Cys Leu
    210   215   220

Ala Val Gly Met Arg Pro Glu Cys Val Val Pro Glu Asn Gln Cys Ala
225   230   235   240

Met Lys Arg Lys Glu Lys Lys Ala Gln Arg Glu Lys Asp Lys Leu Pro
                245   250   255

Val Ser Thr Thr Thr Val Asp Asp His Met Pro Pro Ile Met Gln Cys
            260   265   270

Asp Pro Pro Pro Pro Glu Ala Ala Arg Ile Leu Glu Cys Val Gln His
        275   280   285

Glu Val Val Pro Arg Phe Leu Asn Glu Lys Leu Met Glu Gln Asn Arg
    290   295   300

Leu Lys Asn Val Pro Pro Leu Thr Ala Asn Gln Lys Ser Leu Ile Ala
305   310   315   320

Arg Leu Val Trp Tyr Gln Glu Gly Tyr Glu Gln Pro Ser Glu Glu Asp
                325   330   335

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Leu Lys Arg Val Thr Gln Ser Asp Glu Asp Asp Glu Asp Ser Asp Met
            340                 345                 350

Pro Phe Arg Gln Ile Thr Glu Met Thr Ile Leu Thr Val Gln Leu Ile
        355                 360                 365

Val Glu Phe Ala Lys Gly Leu Pro Gly Phe Ala Lys Ile Ser Gln Ser
    370                 375                 380

Asp Gln Ile Thr Leu Leu Lys Ala Cys Ser Ser Glu Val Met Met Leu
385                 390                 395                     400

Arg Val Ala Arg Arg Tyr Asp Ala Ala Thr Asp Ser Val Leu Phe Ala
                405                 410                 415

Asn Asn Gln Ala Tyr Thr Arg Asp Asn Tyr Arg Lys Ala Gly Met Ala
            420                 425                 430

Tyr Val Ile Glu Asp Leu Leu His Phe Cys Arg Cys Met Tyr Ser Met
        435                 440                 445

Met Met Asp Asn Val His Tyr Ala Leu Leu Thr Ala Ile Val Ile Phe
    450                 455                 460

Ser Asp Arg Pro Gly Leu Glu Gln Pro Leu Leu Val Glu Asp Ile Gln
465                 470                 475                     480

Arg Tyr Tyr Leu Asn Thr Leu Arg Val Tyr Ile Leu Asn Gln Asn Ser
                485                 490                 495

Ala Ser Pro Arg Gly Ala Val Ile Phe Gly Glu Ile Leu Gly Ile Leu
            500                 505                 510

Thr Glu Ile Arg Thr Leu Gly Met Gln Asn Ser Asn Met Cys Ile Ser
        515                 520                 525

Leu Lys Leu Lys Lys Arg Lys Leu Pro Pro Phe Leu Glu Glu Ile Trp
    530                 535                 540

Asp Val Ala Asp Val Ala Thr Thr Ala Thr Pro Val Ala Ala Glu Ala
545                 550                 555                     560

Pro Ala Pro Leu Ala Pro Ala Pro Pro Ala Arg Pro Ala Thr Val
                565                 570                 575
```

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 948 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Spodoptera exigua

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

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AGGCCGGAGT GCGTGGTGCC AGAAAACCAG TGTGCAATGA AAGGAAAGA GAAAAAGGCA        60
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CAAAGGGAAA AAGACAAGTT GCCAGTCAGT ACAACGACAG TGGATGATCA CATGCCTCCC 120

ATTATGCAGT GTGATCCACC GCCTCCAGAG GCCGCAAGAA TTCACGAGGT GGTGCCACGA 180

TTCCTGAATG AAAAGCTAAT GGACAGGACA AGGCTCAAGA ATGTGCCCCC TCACTGCCAA 240

CCAGAAGTCC TTAATAGCGA GGCTGGTCTG GTACCAAGAA GGCTATGAAC AGCCATCAGA 300

AGAGGATCTA AAAAGAGTCA CACAGTCGGA TGAAGACGAA GAAGAGTCGG ACATGCCGTT 360

CCGTCAGATC ACCGAGATGA CGATCCTCAC AGTGCAGCTC ATTGTTGAAT TCGCTAAGGG 420

CCTACCAGCG TTCGCAAAGA TCTCACAGTC GGATCAGATC ACATTATTAA AGGCCTGTTC 480

GAGTGAGGTG ATGATGTTGC GAGTAGCTCG GCGGTACGAC GCGGCGACAG ACAGCGTGTT 540

GTTCGCCAAC AACCAGGCGT ACACCCGCGA CAACTACCGC AAGGCAGGCA TGGCCTACGT 600

CATCGAGGAC CTGCTGCACT TCTGCCGGTG CATGTACTCC ATGATGATGG ATAACGTCCA 660

CTATGCACTG CTCACTGCCA TCGTCATTTT CTCAGACCGA CCCGGGCTTG AGCTAACCCT 720

GTTGGTGGAG GAGATCCAGA GATATTACCT GAACACGCTG CGGGTGTACA TCCTGAACCA 780

GAACAGTCGG TCGCCGTGCT GCCCTGTCAT CTACGCTAAG ATCCTCGGCA TCCTGACGGA 840

GCTGCGGACC CTGGGCATGC AGAACTCCAA CATGTGCATC TCACTCAAGC TGAAGAACAG 900

GAACGTGCCG CCGTTCTTCG AGGATATCTG GGACGTCCTC GAGTAAAA 948

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 319 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

```
Arg Pro Glu Cys Val Val Pro Glu Asn Gln Cys Ala Met Lys Arg Lys
1               5                   10                  15

Glu Lys Lys Ala Gln Arg Glu Lys Asp Lys Leu Pro Val Ser Thr Thr
            20                  25                  30

Thr Val Asp Asp His Met Pro Pro Ile Met Gln Cys Asp Pro Pro Pro
        35                  40                  45

Pro Glu Ala Ala Arg Ile Leu Glu Cys Val Gln His Glu Val Val Pro
    50                  55                  60

Arg Phe Leu Asn Glu Lys Leu Met Glu Gln Asn Arg Leu Lys Asn Val
65                  70                  75                  80

Pro Pro Leu Thr Ala Asn Gln Lys Ser Leu Ile Ala Arg Leu Val Trp
                85                  90                  95
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Tyr Gln Glu Gly Tyr Glu Gln Pro Ser Glu Glu Asp Leu Lys Arg Val
100 105 110

Thr Gln Ser Asp Glu Asp Asp Glu Asp Ser Asp Met Pro Phe Arg Gln
115 120 125

Ile Thr Glu Met Thr Ile Leu Thr Val Gln Leu Ile Val Glu Phe Ala
130 135 140

Lys Gly Leu Pro Gly Phe Ala Lys Ile Ser Gln Ser Asp Gln Ile Thr
145 150 155 160

Leu Leu Lys Ala Cys Ser Ser Glu Val Met Met Leu Arg Val Ala Arg
165 170 175

Arg Tyr Asp Ala Ala Thr Asp Ser Val Leu Phe Ala Asn Asn Gln Ala
180 185 190

Tyr Thr Arg Asp Asn Tyr Arg Lys Ala Gly Met Ala Tyr Val Ile Glu
195 200 205

Asp Leu Leu His Phe Cys Arg Cys Met Tyr Ser Met Met Met Asp Asn
210 215 220

Val His Tyr Ala Leu Leu Thr Ala Ile Val Ile Phe Ser Asp Arg Pro
225 230 235 240

Gly Leu Glu Gln Pro Leu Leu Val Glu Glu Ile Gln Arg Tyr Tyr Leu
245 250 255

Asn Thr Leu Arg Val Tyr Ile Leu Asn Gln Asn Ser Ala Ser Pro Arg
260 265 270

Gly Ala Val Ile Phe Gly Glu Ile Leu Gly Ile Leu Thr Glu Ile Arg
275 280 285

Thr Leu Gly Met Gln Asn Ser Asn Met Cys Ile Ser Leu Lys Leu Lys
290 295 300

Lys Arg Lys Leu Pro Pro Phe Leu Glu Glu Ile Asp Trp Asp Val
305 310 315

CLAIMS

1. DNA comprising the sequence shown in Seq ID No. 2.

2. DNA comprising the sequence shown in Seq ID No. 3.

3. DNA comprising the sequence shown in Seq ID No. 4.

4. DNA comprising a sequence which shows 60% or more homology with the sequence shown in Seq ID No 1, 2 or 3.

5. DNA according to claim 4 wherein said homology is in the range of 65% to 99%.

6. DNA which hybridises to the sequence shown in Seq. ID No. 2, 3 or 4, and which codes for at least part of the *Heliothis* ecdysone receptor.

7. DNA which is degenerate as a result of the genetic code to the DNA of any one of claims 1 to 6 and which codes for a polypeptide which is at least part of the *Heliothis* ecdysone receptor.

8. DNA comprising part of the sequence shown in Seq ID No. 2, and which codes for at least part of the *Heliothis* ecdysone receptor ligand binding domain.

9. DNA comprising part of the sequence shown in Seq ID No. 3, and which codes for at least part of the *Heliothis* ecdysone receptor ligand binding domain.

10. DNA comprising part of the sequence shown in Seq ID No. 4, and which codes for at least part of the *Heliothis* ecdysone receptor ligand binding domain.

11. DNA comprising a sequence which shows 60% or more homology with the sequence of claim 8, 9 or 10.

12. DNA according to claim 11 wherein said homology is in the range of 65% to 99%.

13. DNA which hybridises to the DNA of any one of claims 8 to 12 and which codes for at least part of the *Heliothis* ecdysone receptor ligand binding domain.

14. DNA which is degenerate as a result of the genetic code to the DNA of any one of claims 8 to 12 and which codes for a polypeptide which is at least part of the *Heliothis* ecdysone receptor ligand binding domain.

15. DNA comprising part of the sequence shown in Seq ID No. 2, and which codes for at least part of the *Heliothis* ecdysone receptor DNA binding domain.

16. DNA comprising part of the sequence shown in Seq ID No. 3, and which codes for at least part of the *Heliothis* ecdysone receptor DNA binding domain.

17. DNA comprising part of the sequence shown in Seq ID No. 4, and which codes for at least part of the *Heliothis* ecdysone receptor DNA binding domain.

18. DNA comprising a sequence which shows 60% or more homology with the sequence of claim 15, 16 or 17.

19. DNA according to claim 18 wherein said homology is in the range of 65% to 99%.

20. DNA which hybridises to the DNA of any one of claims 15 to 19 and which codes for at least part of the *Heliothis* ecdysone receptor DNA binding domain.

21. DNA which is degenerate as a result of the genetic code to the DNA of any one of claims 15 to 19 and which codes for a polypeptide which is at least part of the *Heliothis* ecdysone receptor DNA binding domain.

22. DNA comprising part of the sequence shown in Seq ID No. 2, and which codes for at least part of the *Heliothis* ecdysone receptor transactivation domain.

23. DNA comprising part of the sequence shown in Seq ID No. 3, and which codes for at least part of the *Heliothis* ecdysone receptor transactivation domain.

24. DNA comprising part of the sequence shown in Seq ID No. 4, and which codes for at least part of the *Heliothis* ecdysone receptor transactivation domain.

25. DNA comprising a sequence which shows 60% or more homology with the sequence of claim 22, 23 or 24.

26. DNA according to claim 25 wherein said homology is in the range of 65% to 99%.

27. DNA which hybridises to the DNA of any one of claims 22 to 26 and which codes for at least part of the *Heliothis* ecdysone receptor transactivation domain.

28. DNA which is degenerate as a result of the genetic code to the DNA of any one of claims 22 to 26 and which codes for a polypeptide which is at least part of the *Heliothis* ecdysone receptor transactivation domain.

29. DNA comprising part of the sequence shown in Seq ID No. 2, and which codes for at least part of the *Heliothis* ecdysone receptor hinge domain.

30. DNA comprising part of the sequence shown in Seq ID No. 3, and which codes for at least part of the *Heliothis* ecdysone receptor hinge domain.

31. DNA comprising part of the sequence shown in Seq ID No. 4, and which codes for at least part of the *Heliothis* ecdysone receptor hinge domain.

32. DNA comprising a sequence which shows 60% or more homology with the sequence of claim 29, 30 or 31.

33. DNA according to claim 32 wherein said homology is in the range of 65% to 99%.

34. DNA which hybridises to the DNA of any one of claims 29 to 33 and which codes for at least part of the *Heliothis* ecdysone receptor hinge domain.

35. DNA which is degenerate as a result of the genetic code of the DNA of any one of claims 29 to 33 and which codes for a polypeptide which is at least part of the *Heliothis* ecdysone receptor hinge domain.

36. DNA having part of the sequence shown in Seq ID No. 2, and which codes for at least part of the *Heliothis* ecdysone receptor carboxy terminal region.

37. DNA having part of the sequence shown in Seq ID No. 3, and which codes for at least part of the *Heliothis* ecdysone receptor carboxy terminal region.

38. DNA having part of the sequence shown in Seq ID No. 4, and which codes for at least part of the *Heliothis* ecdysone receptor carboxy terminal region.

39. DNA comprising a sequence which shows 60% or more homology with the sequence of claim 36, 37 or 38.

40. DNA according to claim 39 wherein said homology is in the range of 65% to 99%.

41. DNA which hybridises to the DNA of any one of claims 36 to 40 and which codes for at least part of the *Heliothis* ecdysone receptor carboxy terminal region.

42. DNA which is degenerate as a result of the genetic code of the DNA of any one of claims 36 to 40 and which codes for a polypeptide which is at least part of the *Heliothis* ecdysone receptor carboxy terminal region.

43. A polypeptide comprising the *Heliothis* ecdysone receptor or a fragment thereof, wherein said polypeptide is substantially free from other proteins with which it is ordinarily associated, and which is coded for by the DNA of any preceding claim.

44. A polypeptide comprising the amino acid sequence shown in Seq ID No. 4 or any allelic variant or derivative thereof.

45. A polypeptide comprising part of the amino acid sequence shown in Seq ID No. 4 or any allelic variant or derivative thereof, which sequence provides the *Heliothis* ecdysone receptor ligand binding domain.

46. A polypeptide comprising part of the amino acid sequence shown in Seq ID No. 4 or any allelic variant or derivative thereof, which sequence provides the *Heliothis* ecdysone receptor DNA binding domain.

47. A polypeptide comprising part of the amino acid sequence shown in Seq ID No. 4 or any allelic variant or derivative thereof, which sequence provides the *Heliothis* ecdysone receptor transactivation domain.

48. A polypeptide comprising part of the amino acid sequence shown in Seq ID No. 4 or any allelic variant or derivative thereof, which sequence provides the *Heliothis* ecdysone receptor hinge domain.

49. A polypeptide comprising part of the amino acid sequence shown in Seq ID No. 4 or any allelic variant or derivative thereof, which sequence provides the *Heliothis* ecdysone receptor carboxy terminal region.

50. A polypeptide according to any one of claims 44 to 49 wherein said derivative is a homologous variant which includes conservative amino acid changes.

51. DNA comprising the sequence shown in Seq ID No. 6.

52. DNA comprising a sequence which shows 60% or more homology with the sequence shown in Seq ID No. 6.

53. DNA according to claim 52 wherein said homology is in the range of 65% to 99%.

54. DNA which hybridises to the DNA sequence shown in Seq ID No. 6 and which codes for at least part of *Spodoptera* ecdysone receptor.

55. DNA which is degenerate as a result of the genetic code to the DNA of any one of claims 51 to 54.

56. DNA comprising part of the sequence shown in Seq ID No. 6, and which codes for at least part of the *Spodoptera* ecdysone receptor ligand binding domain.

57. DNA comprising a sequence which shows 60% or more homology with the sequence of claim 56.

58. DNA according to claim 57 wherein said homology is in the range of 65% to 99%.

59. DNA which hybridises to the DNA of any one of claims 56 to 58 and which codes for at least part of the *Spodoptera* ecdysone receptor ligand binding domain.

60. DNA which is degenerate as a result of the genetic code to the DNA of any one of claims 56 to 58 and which codes for at least part of the *Spodoptera* ecdysone receptor ligand binding domain.

61. DNA comprising part of the sequence shown in Seq ID No. 6, and which codes for at least part of the *Spodoptera* ecdysone receptor hinge domain.

62. DNA comprising a sequence which shows 60% or more homology with the sequence of claim 61.

63. DNA according to claim 62 wherein said homology is in the range of 65% to 99%.

64. DNA which hybridises to the DNA of any one of claims 61 to 63 and which codes for at least part of the *Spodoptera* ecdysone receptor hinge domain.

65. DNA which is degenerate as a result of the genetic code to the DNA of any one of claims 61 to 63 and which codes for at least part of the *Spodoptera* ecdysone receptor hinge domain.

66. A polypeptide coded for by the DNA of any one of claims 51 to 65.

67. A fusion polypeptide comprising the polypeptide of claim 45 or 50 (when dependent upon claim 45) and functionally linked to a DNA binding domain and a transactivation domain.

68. Recombinant DNA comprising the DNA of any one of claim 8 to 14 functionally linked to DNA encoding a DNA binding domain and a transactivation domain.

69. A fusion polypeptide according to claim 67 or recombinant DNA according to claim 68 wherein the DNA binding domain and/or transactivation domain is fungal, bacterial, plant or mammalian.

70. A fusion polypeptide or recombinant DNA according to claim 69 wherein the DNA binding domain is GAL4 or A1CR/A.

71. A fusion polypeptide or recombinant DNA according to claim 69 or 70 wherein the transactivation domain is VP16.

72. A fusion polypeptide or recombinant DNA according to claim 69 wherein the DNA binding domain and/or transactivation domain is from a steroid receptor superfamily member.

73. A fusion polypeptide or recombinant DNA according to claim 72 wherein the DNA binding domain and/or transactivation domain is from a glucocorticoid or a *Spodoptera* ecdysone receptor.

74. A recombinant DNA construct comprising recombinant DNA of any one of claims 68 to 73; and DNA which codes for a gene operably linked to a promoter sequence and a hormone response element, which is responsive to the DNA binding domain coded for by said recombinant DNA.

75. A fusion polypeptide comprising the polypeptide of claim 46 or 50 (when dependent upon claim 46) and functionally linked to a ligand binding domain and a transactivation domain.

76. Recombinant DNA comprising the DNA of any of claims 15 to 21 functionally linked to DNA encoding a ligand binding domain and a transactivation domain.

77. A fusion polypeptide according to claim 75 or recombinant DNA according to claim 76 wherein the ligand binding domain and/or transactivation domain is fungal, bacterial, plant or mammalian.

78. A fusion polypeptide or recombinant DNA according to claim 77 wherein the transactivation domain is VP16.

79. A fusion polypeptide or recombinant DNA according to claim 77 wherein the ligand binding domain and/or transactivation domain is from a steroid receptor superfamily member.

80. A fusion polypeptide or recombinant DNA according to claim 79 wherein the ligand binding domain and/or transactivation domain is from a glucocorticoid or *Spodoptera* ecdysone receptor.

81. A recombinant DNA construct comprising recombinant DNA of any one of claims 76 to 80; and DNA which codes for a gene operably linked to a promoter sequence and a hormone response element, which is responsive to the DNA binding domain coded for by said recombinant DNA.

82. A fusion polypeptide comprising the polypeptide of claim 47 or 50 (when dependent upon claim 47) and functionally linked to a ligand binding domain and a DNA binding domain.

83. Recombinant DNA comprising the DNA of any one of claims 22 to 28 functionally linked to DNA encoding a ligand binding domain and a DNA binding domain.

84. A fusion polypeptide according to claim 82 or recombinant DNA according to claim 83 wherein the ligand binding domain and/or DNA binding domain is fungal, bacterial, plant or mammalian.

85. A fusion polypeptide or recombinant DNA according to claim 84 wherein the DNA binding domain is GAL4 or A1CR/A.

86. A fusion polypeptide or recombinant DNA according to claim 84 wherein the ligand binding domain and/or DNA binding domain is from a steroid receptor superfamily member.

87. A fusion polypeptide or recombinant DNA according to claim 86 wherein the ligand binding domain and/or DNA binding domain is from a glucocorticoid or *Spodoptera* ecdysone receptor.

88. A recombinant DNA construct comprising recombinant DNA of any one of claims 82 to 87; and DNA which codes for a gene operably linked to a promoter sequence and a hormone response element, which is responsive to the DNA binding domain coded for by said recombinant DNA.

89. A recombinant DNA construct comprising DNA according to any one of claims 1 to 7; and DNA comprising a sequence which codes for a gene operably linked to a promoter sequence and at least one hormone response element which is responsive to the DNA binding domain coded for by said DNA of any one of claim 1 to 7.

90. A recombinant DNA construct according to any one of claims 74, 81, 88 and 89 wherein said promoter sequence codes for a constitutive, spatially or temporally regulating promoter.

91. A recombinant DNA construct according to any one of claims 74, 81, 88 and 89 wherein there is more than one copy of the hormone response element.

92. A cell transformed with the DNA of any one of claims 1 to 42, and 51 to 65; the polypeptide of any one of claims 43 to 50; the fusion polypeptide of any one of claims 67, 70 to 73, 75, 77 to 80, 82 and 84 to 87; the recombinant nucleic acid of any one of claims 68 to 73, 76 to 80 and 85 to 87; or the recombinant DNA construct of any one of claims 74, 81, 88 and 89.

93. A cell according to claim 92 wherein said cell is a plant, fungal or mammalian cell.

94. A plant, fungus or mammal comprising the recombinant DNA construct of any one of claims 74, 81, 88 and 89.

95. A method of selecting compounds capable of being bound to an insect steroid receptor superfamily member comprising screening compounds for binding to said polypeptide of any one of claims 43 to 50 or the fusion polypeptide of any one of claims 67, 70 to 73, 75, 77 to 80, 82 and 84 to 87, and selecting said compounds exhibiting said binding.

96. A compound selected using the method of claim 95.

97. An agricultural or pharmaceutical composition comprising the compound of claim 96.

98. Use of the compound of claim 96 as an agrochemical or a pharmaceutical.

99. A method of producing a protein, peptide or polypeptide comprising introducing into the cell of claim 92, a compound which binds to the ligand binding domain in said cell.

Fig.1.

Sequence ID 1

```
1   TGCG AGG GGT GCA AGG AGT TCT TCA GGC GGA GTG TAA CCA AAA ATG
    ACGC TCC CCA CGT TCC TCA AGA AGT CCG CCT CAC ATT GGT TTT TAC

46  CAG TGT ACA TAT GCA AAT TCG GCC ATG CTT GCG AAA TGG ATA TGT
    GTC ACA TGT ATA CGT TTA AGC CGG TAC GAA CGC TTT ACC TAT ACA

91  ATA TGC GGA GAA AAT GCC AAG AGT A
    TAT ACG CCT CTT TTA CGG TTC TCA T
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Fig.2.

Sequence ID 2

	3		9		15		21		27		33		39		45
1	TCC	ACT	GGT	GTT	TTC	ACC	ACC	ACA	GAA	AAG	GCC	TCT	GCT	CAT	TTA
	AGG	TGA	CCA	CAA	AAG	TGG	TGG	TGT	CTT	TTC	CGG	AGA	CGA	GTA	AAT
46	GAG	GGT	GGT	GCT	AAG	AAG	GTC	ATC	ATC	TCC	TGC	TGC	CCA	GCG	CTG
	CTC	CCA	CCA	CGA	TTC	TTC	CAG	TAG	TAG	AGG	ACG	ACG	GGT	CGC	GAC
91	ACC	CAT	GTT	CGT	CGT	TGG	TGT	CAA	CCT	TGA	AGC	AGT	ATG	ACC	CCT
	TGG	GTA	CAA	GCA	GCA	ACC	ACA	GTT	GGA	ACT	TCG	TCA	TAC	TGG	GGA
136	CTT	ACA	AGG	TCA	TCT	CCA	ACG	CCT	CCT	GCA	CAA	CCA	ACT	GCC	TCG
	GAA	TGT	TCC	AGT	AGA	GGT	TGC	GGA	GGA	CGT	GTT	GGT	TGA	CGG	AGC
181	CTC	CTC	TCG	CTA	AGG	TCA	TCC	ATG	ACA	ACT	TCG	AGA	TCA	TTG	AAG
	GAG	GAG	AGC	GAT	TCC	AGT	AGG	TAC	TGT	TGA	AGC	TCT	AGT	AAC	TTC
226	GTC	TGA	TGA	CCA	CTG	TAC	ACG	CCA	CCA	CTG	CCA	CCC	AGA	AGA	CAG
	CAG	ACT	ACT	GGT	GAC	ATG	TGC	GGT	GGT	GAC	GGT	GGG	TCT	TCT	GTC
271	TGG	ATG	GAC	CCT	CTG	GTA	AAC	TGT	GGC	GTG	ATG	GCC	GTG	GTG	CTC
	ACC	TAC	CTG	GGA	GAC	CAT	TTG	ACA	CCG	CAC	TAC	CGG	CAC	CAC	GAG
316	AGC	AGA	ATA	TCA	TTC	CCG	CGG	AAT	TCC	CCA	GCC	GCA	GCT	AGC	TAA
	TCG	TCT	TAT	AGT	AAG	GGC	GCC	TTA	AGG	GGT	CGG	CGT	CGA	TCG	ATT

Fig.2 i.

```
361 CCT GCA GCA GAC ACA ACC CCT ACC TTC CAT GCC GTT ACC AAT GCC
    GGA CGT CGT CTG TGT TGG GGA TGG AAG GTA CGG CAA TGG TTA CGG

406 ACC GAC AAC ACC CAA ATC AGA AAA CGA GTC AAT GTC ATC AGG TCG
    TGG CTG TTG TGG GTT TAG TCT TTT GCT CAG TTA CAG TAG TCC AGC

451 TGA GGA ACT GTC TCC AGC TTC GAG TGT AAA CGG CTG CAG CAC AGA
    ACT CCT TGA CAG AGG TCG AAG CTC ACA TTT GCC GAC GTC GTG TCT

496 TGG CGA GGC GAG GCG GCA GAA GAA AGG CCC AGC GCC GAG GCA GCA
    ACC GCT CCG CTC CGC CGT CTT CTT TCC GGG TCG CGG CTC CGT CGT

541 AGA AGA GCT ATG TCT TGT CTG CGG CGA CAG AGC CTC CGG ATA TCA
    TCT TCT CGA TAC AGA ACA GAC GCC GCT GTC TCG GAG GCC TAT AGT

586 CTA CAA CGC GCT CAC ATG TGA AGG GTG TAA AGG TTT CTT CAG GCG
    GAT GTT GCG CGA GTG TAC ACT TCC CAC ATT TCC AAA GAA GTC CGC

631 GAG TGT AAC CAA AAA TGC AGT GTA CAT ATG CAA ATT CGG CCA TGC
    CTC ACA TTG GTT TTT ACG TCA CAT GTA TAC GTT TAA GCC GGT ACG

676 TTG CGA AAT GGA TAT CTA TAT GCG GAG AAA ATG TCA GGA GTG TCG
    AAC GCT TTA CCT ATA GAT ATA CGC CTC TTT TAC AGT CCT CAC AGC

721 GTT GAA GAA ATG TCT TGC GGT GGG CAT GAG GCC CGA GTG CGT GGT
    CAA CTT CTT TAC AGA ACG CCA CCC GTA CTC CGG GCT CAC GCA CCA

766 GCC GGA GAA CCA GTG TGC AAT GAA ACG GAA AGA GAA AAA GGC GCA
    CGG CCT CTT GGT CAC ACG TTA CTT TGC CTT TCT CTT TTT CCG CGT
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Fig.2 ii.

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811  GAG GGA AAA AGA CAA ATT GCC CGT CAG TAC GAC GAC AGT AGA CGA
     CTC CCT TTT TCT GTT TAA CGG GCA GTC ATG CTG CTG TCA TCT GCT

856  TCA CAT GCC TCC CAT CAT GCA ATG TGA CCC TCC GCC CCC AGA GGC
     AGT GTA CGG AGG GTA GTA CGT TAC ACT GGG AGG CGG GGG TCT CCG

901  CGC TAG AAT TCT GGA ATG TGT GCA GCA CGA GGT GGT GCC ACG ATT
     GCG ATC TTA AGA CCT TAC ACA CGT CGT GCT CCA CCA CGG TGC TAA

946  CCT GAA TGA GAA GCT AAT GGA ACA GAA CAG ATT GAA GAA CGT GCC
     GGA CTT ACT CTT CGA TTA CCT TGT CTT GTC TAA CTT CTT GCA CGG

991  CCC CCT CAC TGC CAA TCA GAA GTC GTT GAT CGC AAG GCT CGT GTG
     GGG GGA GTG ACG GTT AGT CTT CAG CAA CTA GCG TTC CGA GCA CAC

1036 GTA CCA GGA AGG CTA TGA ACA ACC TTC CGA GGA AGA CCT GAA GAG
     CAT GGT CCT TCC GAT ACT TGT TGG AAG GCT CCT TCT GGA CTT CTC

1081 GGT TAC ACA GTC GGA CGA GGA CGA CGA AGA CTC GGA TAT GCC GTT
     CCA ATG TGT CAG CCT GCT CCT GCT GCT TCT GAG CCT ATA CGG CAA

1126 CCG TCA GAT TAC CGA GAT GAC GAT TCT CAC AGT GCA GCT CAT CGT
     GGC AGT CTA ATG GCT CTA CTG CTA AGA GTG TCA CGT CGA GTA GCA

1171 AGA ATT CGC TAA GGG CCT CCC GGG CTT CGC CAA GAT CTC GCA GTC
     TCT TAA GCG ATT CCC GGA GGG CCC GAA GCG GTT CTA GAG CGT CAG

1216 GGA CCA GAT CAC GTT ATT AAA GGC GTG CTC AAG TGA GGT GAT GAT
     CCT GGT CTA GTG CAA TAA TTT CCG CAC GAG TTC ACT CCA CTA CTA

1261 GCT CCG AGT GGC TCG GCG GTA TGA CGC GGC CAC CGA CAG CGT ACT
     CGA GGC TCA CCG AGC CGC CAT ACT GCG CCG GTG GCT GTC GCA TGA
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Fig.2 iii.

```
1306 GTT CGC GAA CAA CCA GGC GTA CAC TCG CGA CAA CTA CCG CAA GGC
     CAA GCG CTT GTT GGT CCG CAT GTG AGC GCT GTT GAT GGC GTT CCG

1351 AGG CAT GGC GTA CGT CAT CGA GGA CCT GCT GCA CTT CTG TCG GTG
     TCC GTA CCG CAT GCA GTA GCT CCT GGA CGA CGT GAA GAC AGC CAC

1396 CAT GTA CTC CAT GAT GAT GGA TAA CGT GCA TTA TGC GCT GCT TAC
     GTA CAT GAG GTA CTA CTA CCT ATT GCA CGT AAT ACG CGA CGA ATG

1441 AGC CAT TGT CAT CTT CTC AGA CCG GCC CGG GCT TGA GCA ACC CCT
     TCG GTA ACA GTA GAA GAG TCT GGC CGG GCC CGA ACT CGT TGG GGA

1486 GTT GGT GGA GGA CAT CCA GAG ATA TTA CCT GAA CAC GCT ACG GGT
     CAA CCA CCT CCT GTA GGT CTC TAT AAT GGA CTT GTG CGA TGC CCA

1531 GTA CAT CCT GAA CCA GAA CAG CGC GTC GCC CCG CGG CGC CGT CAT
     CAT GTA GGA CTT GGT CTT GTC GCG CAG CGG GGC GCC GCG GCA GTA

1576 CTT CGG CGA GAT CCT GGG CAT ACT GAC GGA GAT CCG CAC GCT GGG
     GAA GCC GCT CTA GGA CCC GTA TGA CTG CCT CTA GGC GTG CGA CCC

1621 CAT GCA GAA CTC CAA CAT GTG CAT CTC CCT CAA GCT GAA GAA CAG
     GTA CGT CTT GAG GTT GTA CAC GTA GAG GGA GTT CGA CTT CTT GTC

1666 GAA GCT GCC GCC GTT CCT CGA GGA GAT CTG GGA CGT GGC GGA CGT
     CTT CGA CGG CGG CAA GGA GCT CCT CTA GAC CCT GCA CCG CCT GCA

1711 GGC GAC GAC GGC GAC GCC GGT GGC GGC GGA GGC GCC GGC GCC TCT
     CCG CTG CTG CCG CTG CGG CCA CCG CCG CCT CCG CGG CCG CGG AGA
```

Fig.2 iv.

```
1756 AGC CCC CGC CCC GCC CGC CCG GCC GCC CGC CAC CGT CTA GCG CGC
     TCG GGG GCG GGG CGG GCG GGC CGG CGG GCG GTG GCA GAT CGC GCG

1801 CTC AGG AGA GAA CGC TCA TAG ACT GGC TAG TTT TAG TGA AGT GCA
     GAG TCC TCT CTT GCG AGT ATC TGA CCG ATC AAA ATC ACT TCA CGT

1846 CGG ACA CTG ACG TCG ACG TGA TCA ACC TAT TTA TAA GGA CTG CGA
     GCC TGT GAC TGC AGC TGC ACT AGT TGG ATA AAT ATT CCT GAC GCT

1891 ATT TTA CCA CTT AAG AGG GCA CAC CCG TAC CCG ATT TCG TAC GG
     TAA AAT GGT GAA TTC TCC CGT GTG GGC ATG GGC TAA AGC ATG CC
```

Total number of bases is: 1934.

Fig.3.

The sequence shown below is that of pSK16.1

Sequence ID3

```
        3           9          15          21          27          33          39          45
        |           |           |           |           |           |           |           |
  1   CGC TGG TAT AAC AAC GGA CCA TTC CAG ACG CTG CGA ATG CTC GAG
      GCG ACC ATA TTG TTG CCT GGT AAG GTC TGC GAC GCT TAC GAG CTC

 46   GAG AGC TCG TCT GAG GTG ACG TCG TCT TCA GCA CTG GGC CTG CCG
      CTC TCG AGC AGA CTC CAC TGC AGC AGA AGT CGT GAC CCG GAC GGC

 91   CCG GCT ATG GTG ATG TCC CCG GAA TCG CTC GCG TCG CCC GAG ATC
      GGC CGA TAC CAC TAC AGG GGC CTT AGC GAG CGC AGC GGG CTC TAG

136   GGC GGC CTG GAG CTG TGG GGC TAC GAC GAT GGC ATC ACT TAC AGC
      CCG CCG GAC CTC GAC ACC CCG ATG CTG CTA CCG TAG TGA ATG TCG

181   ATG GCA CAG TCG CTG GGC ACC TGC ACC ATG GAG CAG CAG CAG CCC
      TAC CGT GTC AGC GAC CCG TGG ACG TGG TAC CTC GTC GTC GTC GGG
```

Fig.3 i.

```
226 CAG CCG CAG CAG CAG CCG CAG CAG ACA CAA CCC CTA CCT TCC ATG
    GTC GGC GTC GTC GTC GGC GTC GTC TGT GTT GGG GAT GGA AGG TAC

271 CCG TTA CCA ATG CCA CCG ACA ACA CCC AAA TCA GAA AAC GAG TCA
    GGC AAT GGT TAC GGT GGC TGT TGT GGG TTT AGT CTT TTG CTC AGT

316 ATG TCA TCA GGT CGT GAG GAA CTG TCT CCA GCT TCG AGT GTA AAC
    TAC AGT AGT CCA GCA CTC CTT GAC AGA GGT CGA AGC TCA CAT TTG

361 GGC TGC AGC ACA GAT GGC GAG GCG AGG CGG CAG AAG AAA GGC CCA
    CCG ACG TCG TGT CTA CCG CTC CGC TCC GCC GTC TTC TTT CCG GGT

406 GCG CCG AGG CAG CAA GAA GAG CTA TGT CTT GTC TGC GGC GAC AGA
    CGC GGC TCC GTC GTT CTT CTC GAT ACA GAA CAG ACG CCG CTG TCT

451 GCC TCC GGA TAT CAC TAC AAC GCG CTC ACA TGT GAA GGG TGT AAA
    CGG AGG CCT ATA GTG ATG TTG CGC GAG TGT ACA CTT CCC ACA TTT

496 GGT TTC TTC AGG CGG AGT GTA ACC AAA AAT GCA GTG TAC ATA TGC
```

Fig.3 ii.

```
    CCA AAG AAG TCC GCC TCA CAT TGG TTT TTA CGT CAC ATG TAT ACG

541 AAA TTC GGC CAT GCT TGC GAA ATG GAT ATC TAT ATG CGG AGA AAA
    TTT AAG CCG GTA CGA ACG CTT TAC CTA TAG ATA TAC GCC TCT TTT

586 TGT CAG GAG TGT CGG TTG AAG AAA TGT CTT GCG GTG GGC ATG AGG
    ACA GTC CTC ACA GCC AAC TTC TTT ACA GAA CGC CAC CCG TAC TCC

631 CCC GAG TGC GTG GTG CCG GAG AAC CAG TGT GCA ATG AAA CGG AAA
    GGG CTC ACG CAC CAC GGC CTC TTG GTC ACA CGT TAC TTT GCC TTT

676 GAG AAA AAG GCG CAG AGG GAA AAA GAC AAA TTG CCC GTC AGT ACG
    CTC TTT TTC CGC GTC TCC CTT TTT CTG TTT AAC GGG CAG TCA TGC

721 ACG ACA GTA GAC GAT CAC ATG CCT CCC ATC ATG CAA TGT GAC CCT
    TGC TGT CAT CTG CTA GTG TAC GGA GGG TAG TAC GTT ACA CTG GGA

766 CCG CCC CCA GAG GCC GCT AGA ATT CTG GAA TGT GTG CAG CAC GAG
    GGC GGG GGT CTC CGG CGA TCT TAA GAC CTT ACA CAC GTC GTG CTC

811 GTG GTG CCA CGA TTC CTG AAT GAG AAG CTA ATG GAA CAG AAC AGA
    CAC CAC GGT GCT AAG GAC TTA CTC TTC GAT TAC CTT GTC TTG TCT

856 TTG AAG AAC GTG CCC CCC CTC ACT GCC AAT CAG AAG TCG TTG ATC
    AAC TTC TTG CAC GGG GGG GAG TGA CGG TTA GTC TTC AGC AAC TAG

901 GCA AGG CTC GTG TGG TAC CAG GAA GGC TAT GAA CAA CCT TCC GAG
    CGT TCC GAG CAC ACC ATG GTC CTT CCG ATA CTT GTT GGA AGG CTC

946 GAA GAC CTG AAG AGG GTT ACA CAG TCG GAC GAG GAC GAC GAA GAC
    CTT CTG GAC TTC TCC CAA TGT GTC AGC CTG CTC CTG CTG CTT CTG
```

Fig.3 iii.

```
 991 TCG GAT ATG CCG TTC CGT CAG ATT ACC GAG ATG ACG ATT CTC ACA
     AGC CTA TAC GGC AAG GCA GTC TAA TGG CTC TAC TGC TAA GAG TGT

1036 GTG CAG CTC ATC GTA GAA TTC GCT AAG GGC CTC CCG GGC TTC GCC
     CAC GTC GAG TAG CAT CTT AAG CGA TTC CCG GAG GGC CCG AAG CGG

1081 AAG ATC TCG CAG TCG GAC CAG ATC ACG TTA TTA AAG GCG TGC TCA
     TTC TAG AGC GTC AGC CTG GTC TAG TGC AAT AAT TTC CGC ACG AGT

1126 AGT GAG GTG ATG ATG CTC CGA GTG GCT CGG CGG TAT GAC GCG GCC
     TCA CTC CAC TAC TAC GAG GCT CAC CGA GCC GCC ATA CTG CGC CGG

1171 ACC GAC AGC GTA CTG TTC GCG AAC AAC CAG GCG TAC ACT CGC GAC
     TGG CTG TCG CAT GAC AAG CGC TTG TTG GTC CGC ATG TGA GCG CTG

1216 AAC TAC CGC AAG GCA GGC ATG GCG TAC GTC ATC GAG GAC CTG CTG
     TTG ATG GCG TTC CGT CCG TAC CGC ATG CAG TAG CTC CTG GAC GAC

1261 CAC TTC TGT CGG TGC ATG TAC TCC ATG ATG ATG GAT AAC GTG CAT
     GTG AAG ACA GCC ACG TAC ATG AGG TAC TAC TAC CTA TTG CAC GTA

1306 TAT GCG CTG CTT ACA GCC ATT GTC ATC TTC TCA GAC CGG CCC GGG
     ATA CGC GAC GAA TGT CGG TAA CAG TAG AAG AGT CTG GCC GGG CCC

1351 CTT GAG CAA CCC CTG TTG GTG GAG GAC ATC CAG AGA TAT TAC CTG
     GAA CTC GTT GGG GAC AAC CAC CTC CTG TAG GTC TCT ATA ATG GAC

1396 AAC ACG CTA CGG GTG TAC ATC CTG AAC CAG AAC AGC GCG TCG CCC
     TTG TGC GAT GCC CAC ATG TAG GAC TTG GTC TTG TCG CGC AGC GGG

1441 CGC GGC GCC GTC ATC TTC GGC GAG ATC CTG GGC ATA CTG ACG GAG
```

Fig.3 iv.

```
     GCG CCG CGG CAG TAG AAG CCG CTC TAG GAC CCG TAT GAC TGC CTC

1486 ATC CGC ACG CTG GGC ATG CAG AAC TCC AAC ATG TGC ATC TCC CTC
     TAG GCG TGC GAC CCG TAC GTC TTG AGG TTG TAC ACG TAG AGG GAG

1531 AAG CTG AAG AAC AGG AAG CTG CCG CCG TTC CTC GAG GAG ATC TGG
     TTC GAC TTC TTG TCC TTC GAC GGC GGC AAG GAG CTC CTC TAG ACC

1576 GAC GTG GCG GAC GTG GCG ACG ACG GCG ACG CCG GTG GCG GCG GAG
     CTG CAC CGC CTG CAC CGC TGC TGC CGC TGC GGC CAC CGC CGC CTC

1621 GCG CCG GCG CCT CTA GCC CCC GCC CCG CCC GCC CGG CCG CCC GCC
     CGC GGC CGC GGA GAT CGG GGG CGG GGC GGG CGG GCC GGC GGG CGG

1666 ACC GTC TAG CGC GCC TCA GGA GAG AAC GCT CAT AGA CTG GCT AGT
     TGG CAG ATC GCG CGG AGT CCT CTC TTG CGA GTA TCT GAC CGA TCA

1711 TTT AGT GAA GTG CAC GGA CAC TGA CGT CGA CGT GAT CAA CCT ATT
     AAA TCA CTT CAC GTG CCT GTG ACT GCA GCT GCA CTA GTT GGA TAA

1756 TAT AAG GAC TGC GAA TTT TAC CAC TTA AGA GGG CAC ACC CGT ACC
     ATA TTC CTG ACG CTT AAA ATG GTG AAT TCT CCC GTG TGG GCA TGG

1801 CGA TTT CGT ACG TAT TCG GTG ACC GAC GAC GAT GCA GAG CGT GTG
     GCT AAA GCA TGC ATA AGC CAC TGG CTG CTG CTA CGT CTC GCA CAC

1846 TAA TGT GAA TAT ATG TGT TGT TGA ACG ATT TGG AGA ATA TAT ATT
     ATT ACA CTT ATA TAC ACA ACA ACT TGC TAA ACC TCT TAT ATA TAA

1891 GGT GTT GCT GTT CGG GCC CGC ACG CCG TCG CCG GTC GGC GGC GAT
     CCA CAA CGA CAA GCC CGG GCG TGC GGC AGC GGC CAG CCG CCG CTA
```

Fig.3 v.

```
1936 CGC GGC GCC CGC GGC TTC AGT TTT ATT TCG TTT ACG ACT GAG TTG
     GCG CCG CGG GCG CCG AAG TCA AAA TAA AGC AAA TGC TGA CTC AAC

1981 GTC ACT CGG ATA CGA CTG TAT GAT AAG ACT TCG TTC GAT AAG TAC
     CAG TGA GCC TAT GCT GAC ATA CTA TTC TGA AGC AAG CTA TTC ATG

2026 ACC TAC TAA ATT ACA CAT ACG TAC GTA GCT TAC GAG AGT TAT TAG
     TGG ATG ATT TAA TGT GTA TGC ATG CAT CGA ATG CTC TCA ATA ATC

2071 AGA CAA AGA ATA TAA GAA GAA GAT GTT TCT ATT GGG TGA AAA GTT
     TCT GTT TCT TAT ATT CTT CTT CTA CAA AGA TAA CCC ACT TTT CAA

2116 GAT AGT TAT GTT TAT TTA CCA AAA TTA ACA ATA ATA CGT TGA TTA
     CTA TCA ATA CAA ATA AAT GGT TTT AAT TGT TAT TAT GCA ACT AAT

2161 ACC TTT CGA GTA TAA TAT TGT GAT GAG TCG TCC GCT GTC CAC GTC
     TGG AAA GCT CAT ATT ATA ACA CTA CTC AGC AGG CGA CAG GTG CAG

2206 GCC GTC ACA TGT TTG TTT CTG ATG CAC ACG TGA GGN GCG TTA TCG
     CGG CAG TGT ACA AAC AAA GAC TAC GTG TGC ACT CCN CGC AAT AGC

2251 TGT TTC ATG GTT CCA TCG TCC TGT GCC CGC GAC CCT CGA CTA AAT
     ACA AAG TAC CAA GGT AGC AGG ACA CGG GCG CTG GGA GCT GAT TTA

2296 GAG TAA TTT AAT TTA TTG CTG TGA TTA CAT TTT AAT GTG TTG ATT
     CTC ATT AAA TTA AAT AAC GAC ACT AAT GTA AAA TTA CAC AAC TAA

2341 ATC TAC CAT AGG GTG ATA TAA GTG TGT CTT ATT ACA ATA CAA AGT
     TAG ATG GTA TCC CAC TAT ATT CAC ACA GAA TAA TGT TAT GTT TCA

2386 GTG TGT CGT CGA TAG CTT CCA CAC GAG CAA GCC TTT TGT TTA AGT
```

Fig.3 vi.

```
     CAC ACA GCA GCT ATC GAA GGT GTG CTC GTT CGG AAA ACA AAT TCA

2431 GAT TTA CTG ACA TGG ACA CTC GAC CCG GAA CTT C
     CTA AAT GAC TGT ACC TGT GAG CTG GGC CTT GAA G
```

Total number of bases is: 2464.

Fig.4.

Sequence ID 4

```
        10        20        30        40        50        60
         |         |         |         |         |         |
ACTCGCGTGCTCTTCTCACCTGTTGCTCGGATTGTGTTGTACTAGAAAAAAGTTGTCGCC

        70        80        90       100       110       120
         |         |         |         |         |         |
GCTCGAACGAGACTTCCGAGTCCTATTGGATTGCACGAAAGTCGAGACAGTGGATAGCGA

       130       140       150       160       170       180
         |         |         |         |         |         |
TTCGGTTTCGTTTGAACGTTGCGTAGACGAGTGGTGCATGTCCATGAGTCGCGTTTAGAT
```

Fig.4 i.

```
  190       200       210       220       230       240
AGTTTAGTGCGAGGAAAAAGTGAAGTGAAAGCCTTCCTCGGAGGATGTCCCTCGGCGCTC
                                           M  S  L  G  A

  250       260       270       280       290       300
GTGGATACCGGAGGTGTGACACGCTCGCCGACATGAGACGCCGCTGGTATAACAACGGAC
R  G  Y  R  R  C  D  T  L  A  D  M  R  R  R  W  Y  N  N  G

  310       320       330       340       350       360
CATTCCAGACGCTGCGAATGCTCGAGGAGAGCTCGTCTGAGGTGACGTCGTCTTCAGCAC
P  F  Q  T  L  R  M  L  E  E  S  S  S  E  V  T  S  S  S  A

  370       380       390       400       410       420
TGGGCCTGCCGCCGGCTATGGTGATGTCCCCGGAATCGCTCGCGTCGCCCGAGATCGGCG
L  G  L  P  P  A  M  V  M  S  P  E  S  L  A  S  P  E  I  G
```

Fig.4 ii.

```
      430       440       450       460       470       480
        |         |         |         |         |         |
GCCTGGAGCTGTGGGGCTACGACGATGGCATCACTTACAGCATGGCACAGTCGCTGGGCA
 G  L  E  L  W  G  Y  D  D  G  I  T  Y  S  M  A  Q  S  L  G

      490       500       510       520       530       540
        |         |         |         |         |         |
CCTGCACCATGGAGCAGCAGCAGCCCCAGCCGCAGCAGCAGCCGCAGCAGACACAACCCC
 T  C  T  M  E  Q  Q  Q  P  Q  P  Q  Q  Q  P  Q  Q  T  Q  P

      550       560       570       580       590       600
        |         |         |         |         |         |
TACCTTCCATGCCGTTACCAATGCCACCGACAACACCCAAATCAGAAAACGAGTCAATGT
 L  P  S  M  P  L  P  M  P  P  T  T  P  K  S  E  N  E  S  M

      610       620       630       640       650       660
        |         |         |         |         |         |
CATCAGGTCGTGAGGAACTGTCTCCAGCTTCGAGTGTAAACGGCTGCAGCACAGATGGCG
 S  S  G  R  E  E  L  S  P  A  S  S  V  N  G  C  S  T  D  G

      670       680       690       700       710       720
        |         |         |         |         |         |
AGGCGAGGCGGCAGAAGAAAGGCCCAGCGCCGAGGCAGCAAGAAGAGCTATGTCTTGTCT
 E  A  R  R  Q  K  K  G  P  A  P  R  Q  Q  E  E  L  C  L  V
```

Fig.4 iii.

```
      730       740       750       760       770       780
       |         |         |         |         |         |
GCGGCGACAGAGCCTCCGGATATCACTACAACGCGCTCACATGTGAAGGGTGTAAAGGTT

C  G  D  R  A  S  G  Y  H  Y  N  A  L  T  C  E  G  C  K  G

      790       800       810       820       830       840
       |         |         |         |         |         |
TCTTCAGGCGGAGTGTAACCAAAAATGCAGTGTACATATGCAAATTCGGCCATGCTTGCG

F  F  R  R  S  V  T  K  N  A  V  Y  I  C  K  F  G  H  A  C

      850       860       870       880       890       900
       |         |         |         |         |         |
AAATGGATATCTATATGCGGAGAAAATGTCAGGAGTGTCGGTTGAAGAAATGTCTTGCGG

E  M  D  I  Y  M  R  R  K  C  Q  E  C  R  L  K  K  C  L  A

      910       920       930       940       950       960
       |         |         |         |         |         |
TGGGCATGAGGCCCGAGTGCGTGGTGCCGGAGAACCAGTGTGCAATGAAACGGAAAGAGA

V  G  M  R  P  E  C  V  V  P  E  N  Q  C  A  M  K  R  K  E

      970       980       990      1000      1010      1020
       |         |         |         |         |         |
AAAAGGCGCAGAGGGAAAAAGACAAATTGCCCGTCAGTACGACGACAGTAGACGATCACA

K  K  A  Q  R  E  K  D  K  L  P  V  S  T  T  T  V  D  D  H
```

Fig.4 iv.

```
          1030      1040      1050      1060      1070      1080
             |         |         |         |         |         |
TGCCTCCCATCATGCAATGTGACCCTCCGCCCCCAGAGGCCGCTAGAATTCTGGAATGTG
 M  P  P  I  M  Q  C  D  P  P  P  P  E  A  A  R  I  L  E  C

          1090      1100      1110      1120      1130      1140
             |         |         |         |         |         |
TGCAGCACGAGGTGGTGCCACGATTCCTGAATGAGAAGCTAATGGAACAGAACAGATTGA
 V  Q  H  E  V  V  P  R  F  L  N  E  K  L  M  E  Q  N  R  L

          1150      1160      1170      1180      1190      1200
             |         |         |         |         |         |
AGAACGTGCCCCCCCTCACTGCCAATCAGAAGTCGTTGATCGCAAGGCTCGTGTGGTACC
 K  N  V  P  P  L  T  A  N  Q  K  S  L  I  A  R  L  V  W  Y

          1210      1220      1230      1240      1250      1260
             |         |         |         |         |         |
AGGAAGGCTATGAACAACCTTCCGAGGAAGACCTGAAGAGGGTTACACAGTCGGACGAGG
 Q  E  G  Y  E  Q  P  S  E  E  D  L  K  R  V  T  Q  S  D  E
```

Fig.4 v.

```
1270       1280       1290       1300       1310       1320
ACGACGAAGACTCGGATATGCCGTTCCGTCAGATTACCGAGATGACGATTCTCACAGTGC

D  D  E  D  S  D  M  P  F  R  Q  I  T  E  M  T  I  L  T  V

1330       1340       1350       1360       1370       1380
AGCTCATCGTAGAATTCGCTAAGGGCCTCCCGGGCTTCGCCAAGATCTCGCAGTCGGACC

Q  L  I  V  E  F  A  K  G  L  P  G  F  A  K  I  S  Q  S  D

1390       1400       1410       1420       1430       1440
AGATCACGTTATTAAAGGCGTGCTCAAGTGAGGTGATGATGCTCCGAGTGGCTCGGCGGT

Q  I  T  L  L  K  A  C  S  S  E  V  M  M  L  R  V  A  R  R

1450       1460       1470       1480       1490       1500
ATGACGCGGCCACCGACAGCGTACTGTTCGCGAACAACCAGGCGTACACTCGCGACAACT

Y  D  A  A  T  D  S  V  L  F  A  N  N  Q  A  Y  T  R  D  N
```

Fig.4 vi.

```
       1510      1520      1530      1540      1550      1560
          |         |         |         |         |         |
ACCGCAAGGCAGGCATGGCGTACGTCATCGAGGACCTGCTGCACTTCTGTCGGTGCATGT
Y  R  K  A  G  M  A  Y  V  I  E  D  L  L  H  F  C  R  C  M

       1570      1580      1590      1600      1610      1620
          |         |         |         |         |         |
ACTCCATGATGATGGATAACGTGCATTATGCGCTGCTTACAGCCATTGTCATCTTCTCAG
Y  S  M  M  M  D  N  V  H  Y  A  L  L  T  A  I  V  I  F  S

       1630      1640      1650      1660      1670      1680
          |         |         |         |         |         |
ACCGGCCCGGGCTTGAGCAACCCCTGTTGGTGGAGGAGATCCAGAGATATTACCTGAACA
D  R  P  G  L  E  Q  P  L  L  V  E  E  I  Q  R  Y  Y  L  N

       1690      1700      1710      1720      1730      1740
          |         |         |         |         |         |
CGCTACGGGTGTACATCCTGAACCAGAACAGCGCGTCGCCCCGCGGCGCCGTCATCTTCG
T  L  R  V  Y  I  L  N  Q  N  S  A  S  P  R  G  A  V  I  F
```

Fig.4 vii.

```
1750      1760      1770      1780      1790      1800
GCGAGATCCTGGGCATACTGACGGAGATCCGCACGCTGGGCATGCAGAACTCCAACATGT
G  E  I  L  G  I  L  T  E  I  R  T  L  G  M  Q  N  S  N  M

    1810      1820      1830      1840      1850      1860
GCATCTCCCTCAAGCTGAAGAACAGGAAGCTGCCGCCGTTCCTCGAGGAGATCTGGGACG
C  I  S  L  K  L  K  N  R  K  L  P  P  F  L  E  E  I  W  D

    1870      1880      1890      1900      1910      1920
TGGCGGACGTGGCGACGACGGCGACGCCGGTGGCGGCGGAGGCGCCGGCGCCTCTAGCCC
V  A  D  V  A  T  T  A  T  P  V  A  A  E  A  P  A  P  L  A

    1930      1940      1950      1960      1970      1980
CCGCCCCGCCCGCCCGGCCGCCCGCCACCGTCTAGCGCGCCTCAGGAGAGAACGCTCATA
P  A  P  P  A  R  P  P  A  T  V  -

    1990      2000      2010      2020      2030      2040
GACTGGCTAGTTTTAGTGAAGTGCACGGACACTGACGTCGACGTGATCAACCTATTTATA
```

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Fig.4 viii.

```
2050      2060      2070      2080      2090      2100
   |         |         |         |         |         |
AGGACTGCGAATTTTACCACTTAAGAGGGCACACCCGTACCCGATTTCGTACGTATTCGG

2110      2120      2130      2140      2150      2160
   |         |         |         |         |         |
TGACCGACGACGATGCAGAGCGTGTGTAATGTGAATATATGTGTTGTTGAACGATTTGGA

2170      2180      2190      2200      2210      2220
   |         |         |         |         |         |
GAATATATATTGGTGTTGCTGTTCGGCCCGCACGCCGTCGCCGGTCGGCGGCGATCGCG

2230      2240      2250      2260      2270      2280
   |         |         |         |         |         |
GCGCCCGCGGCTTCAGTTTTATTTCGTTTACGACTGAGTTGGTCACTCGGATACGACTGT

2290      2300      2310      2320      2330      2340
   |         |         |         |         |         |
ATGATAAGACTTCGTTCGATAAGTACACCTACTAAATTACACATACGTACGTAGCTTACG

2350      2360      2370      2380      2390      2400
   |         |         |         |         |         |
AGAGTTATTAGAGACAAAGAATATAAGAAGAAGATGTTTCTATTGGGTGAAAAGTTGATA
```

Fig.4 ix.

```
2410      2420      2430      2440      2450      2460
GTTATGTTTATTTACCAAAATTAACAATAATACGTTGATTAACCTTTCGAGTATAATATT

2470      2480      2490      2500      2510      2520
GTGATGAGTCGTCCGCTGTCCACGTCGCCGTCACATGTTTGTTTCTGATGCACACGTGAG

2530      2540      2550      2560      2570      2580
GNCGTTATCGTGTTTCATGGTTCCATCGTCCTGTGCCCGCGACCCTCGACTAAATGAGT

2590      2600      2610      2620      2630      2640
AATTTAATTTATTGCTGTGATTACATTTTAATGTGTTGATTATCTACCATAGGGTGATAT

2650      2660      2670      2680      2690      2700
AAGTGTGTCTTATTACAATACAAAGTGTGTGTCGTCGATAGCTTCCACACGAGCAAGCCT

2710      2720      2730      2740
TTTGTTTAAGTGATTTACTGACATGGACACTCGACCCGGAACTTC
```

Fig.5.

```
Sequence I.D. 5

BmECR      MRVENVDNVS     10
MsECR      ----------
HvECR      M---------      1
CtECR      ----------
AaECR      ----------
DmECR      ----------

BmECR      FALNGRADEWCMSVETRLDSLVREKSEVKAYVGGCPSVITDAGAYDALFD     60
MsECR      --------------------------------------------------
HvECR      -SLGARGYRRC-----------------------------------DTLAD   16
CtECR      --------------------------------------------------
AaECR      --------------------------------------------------
DmECR      --------------------------------------------------

BmECR      M-RRRWSNNGGFP-LRMLEESSSEVTSSSA-LGLPPAMVMSPESLASPEY    107
MsECR      M-RRRWSNNGCFP-LRMFEESSSEVTSSSA-FGMPAAMVMSPESLASPEY     47
HvECR      M-RRRWYNNGGFQTLRMLEESSSEVTSSSA-LGLPPAMVMSPESLASPEI     64
CtECR      M-K-----------------------TENLIVTT-VKVEPLNYASQSF       23
AaECR      MMKRRWSNNGGFTALRMLDDSSSEVTSSSAAL----GMTMSPNSLGSPNY     46
DmECR      M-KRRWSNNGGF--MRLPEESSSEVTSSSNGLVLPSGVNMSPSSLDSHDY     47
           *         .        . .      . ..* . .*..
```

Fig.5 i.

```
BmECR   GALELW-------SY-------------------------------------   114
MsECR   GGLELW-------SY-------------------------------------    55
HvECR   GGLELW-------GY-------------------------------------    72
CtECR   GDNNI---------YGGAT---------------------------------    33
AaECR   DELELW-SSYEDNAYNGHSV--LSNGNNN----LGGCGA-------------    78
DmECR   CDNDKWLCGNESGSFGGSNGHGLSQQQQSVITLAMHGCSSTLPAQTTIIP      97
         . .      .

BmECR   ------------------------------------------DDGITY      121
MsECR   ------------------------------------------DETMTN       61
HvECR   ------------------------------------------DDGIT-       77
CtECR   -----------------------------------KKQRLESDETMNH       46
AaECR   ---------------------------ANNLLMNGIVGNNNL----NGMMN    98
DmECR   INGNANGNGGSTNGQYVPGATNLGALANGMLNGGFNGMQQQIQNGHGLIN    147
                                                   ...

BmECR   NTAQSLLGACNMQQQQLQP-------------QQPHPAPPTLPTMP----    154
MsECR   YPAQSLLGACNAPQQQQQQ-------------QQQQPSAQPLPSMP----     94
HvECR   YSMAQSLGTCTMEQQQPQP-------------QQQPQQTQPLPSMP----    114
CtECR   NQTNMNLESSNMNHNTIS-----GFSSPDVNYEAYSPNSKL-----DDGN     86
AaECR   MASQAVQANANSIQHIVGN-----------LINGVNPNQTLIPPLPS---    134
DmECR   STTPSTPTTPLHLQQNLGGAGGGGIGGMGILHHANGTPNGLIGVVGGGGG    197
             ..    ..          .

BmECR   ------------LPMPPTTPKSENESMSSGREELSPASSINGCSADA--D    190
MsECR   ------------LPMPPTTPKSENESMSSGREELSPASSINGCSTDG--E    130
HvECR   ------------LPMPPTTPKSENESMSSGREELSPASSVNGCSTDG--E    146
CtECR   MSVHMGDG----------------------------LDG-------K        98
AaECR   ---------IIQNTLMNTPRSESVNSISSGREDLSPSSSLNGYT--DGSD    173
DmECR   VGLGVGGGGVGGLGMQHTPRSDSVNSISSGRDDLSPSSSLNGYSANESCD    247
                                              ..*     .
```

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Fig.5 ii.

```
BmECR   ARRQKKGPAPRQQEELCLVCGDRASGYHYNALTCEGCKGFFRRSVTKNAV   240
MsECR   PRRQKKGPAPRQQEELCLVCGDRASGYHYNALTCEGCKGFFRRSVTKNAV   180
HvECR   ARRQKKGPAPRQQEELCLVCGDRASGYHYNALTCEGCKGFFRRSVTKNAV   196
CtECR   KSSSKKGPVPRQQEELCLVCGDRASGYHYNALTCEGCKGFFRRSVTKNAV   148
AaECR   AKKQKKGPTPRQQEELCLVCGDRASGYHYNALTCEGCKGFFRRSVTKNAV   223
DmECR   AKKSKKGPAPRVQEELCLVCGDRASGYHYNALTCEGCKGFFRRSVTKSAV   297
        ..****.** ***********************************.**

BmECR   YICKFGHACEMDMYMRRKCQECRLKKCLAVGMRPECVIQEPS-KNKDRQR   289
MsECR   YICKFGHACEMDMYMRRKCQECRLKKCLAVGMRPECVVPESTCKNKRREK   230
HvECR   YICKFGHACEMDIYMRRKCQECRLKKCLAVGMRPECVVPENQCAMKRKEK   246
CtECR   YCCKFGHECEMDMYMRRKCQECRLKKCLAVGMRPECVVPENQCAIKRKEK   198
AaECR   YCCKFGHACEMDMYMRRKCQECRLKKCLAVGMRPECVVPENQCAIKRKEK   273
DmECR   YCCKFGRACEMDMYMRRKCQECRLKKCLAVGMRPGCVVPGNQCAMKRREK   347
        * ****..****.*********************.**...     *  ...

BmECR   QKKDKGILLPVSTTTV----------------------EDHMPPIMQC   315
MsECR   EAQREKDKLPVSTTTV----------------------DDHMPAIMQC   256
HvECR   KAQREKDKLPVSTTTV----------------------DDHMPPIMQC   272
CtECR   KAQKEKDKVPGIVGSNTSSSSLLNQSLNNGSLKNLEISYREELLQQLMKC   248
AaECR   KAQKEKDKVQTNAT---------VSTTNSTY-RS-------EILPILMKC   306
DmECR   KAQKEKDKMTTSPSSQHGGNGSLASGGGQDFVKK-------EILD-LMTC   389
        . . .  .  .                             . .  .* *

BmECR   DPPPPEAARI-----HEVVPRYLSEKLMEQNRQKNIPPLSANQKSLIARL   360
MsECR   DPPPPEAARI-----HEVVPRFLTEKLMEQNRLKNVTPLSANQKSLIARL   301
HvECR   DPPPPEAARILECVQHEVVPRFLNEKLMEQNRLKNVPPLTANQKSLIARL   322
CtECR   DPPPHPMQQLL------------PEKLLMENRAKGTPQLTANQVAVIYKL   286
AaECR   DPPPHQAIPLL------------PEKLLQENRLRNIPLLTANQMAVIYKL   344
DmECR   EPPQHATIPLL------------PDEILAKCQARNIPSLTYNQLAVITKL   427
        .**..   ..             .... . . .... *. ** ..* .*
```

Fig.5 iii.

```
BmECR   VWYQEGYEQPSDEDLKRVTQTWQ-SDEEDEESDLPFRQITEMTILTVQLI   409
MsECR   VMYQEGYEQPSEEDLKRVTQTWQLEEEEEEETDMPFRQITEMTILTVQLI   351
HvECR   VWYQEGYEQPSEEDLKRVTQS----DEDDEDSDMPFRQITEMTILTVQLI   368
CtECR   IWYQDGYEQPSEEDLKRITTE--LEEEEDQEHEANFRYITEVTILTVQLI   334
AaECR   IWYQDGYEQPSEEDLKRIMIG--SPNEEEDQHDVHFRHITEITILTVQLI   392
DmECR   IWYQDGYEQPSEEDLRRIM-S--QPDENESQTDVSFRHITEITILTVQLI   474
        . **.******.***.*.  .     .*.. . .  ** ***.********

BmECR   VEFAKGLPGFSKISQSDQITLLKASSSEVMMLRVARRYDAASDSVLFANN   459
MsECR   VEFAKGLPGFSKISQSDQITLLKASSSEVMMLRVARRYDAATDSVLFANN   401
HvECR   VEFAKGLPGFAKISQSDQITLLKACSSEVMMLRVARRYDAATDSVLFANN   418
CtECR   VEFAKGLPAFIKIPQEDQITLLKACSSEVMMLRMARRYDHDSDSILFANN   384
AaECR   VEFAKGLPAFTKIPQEDQITLLKACSSEVMMLRMARRYDAATDSILFANN   442
DmECR   VEFAKGLPAFTKIPQEDQITLLKACSSEVMMLRMARRYDHSSDSIFFANN   524
        ********.* **.*.********.********.*****  ..**..****

BmECR   KAYTRDNYRQGGMAYVIEDLLHFCRCMFAMGMDNVHFALLTAIVIFSDRP   509
MsECR   QAYTRDNYRKAGMSYVIEDLLHFCRCMYSMSMDNVHYALLTAIVIFSDRP   451
HvECR   QAYTRDNYRKAGMAYVIEDLLHFCRCMYSMMMDNVHYALLTAIVIFSDRP   468
CtECR   TAYTKQTYQLAGMEETIDDLLHFCRQMYALSIDNVETALLTAIVIFSDRP   434
AaECR   RSYTRDSYRMAGMADTIEDLLHFCRQMFSLTVDNVEYALLTAIVIFSDRP   492
DmECR   RSYTRDSYKMAGMADNIEDLLHFCRQMFSMKVDNVEYALLTAIVIFSDRP   574
         .**...*. .**.  *.******* *... .***. *************

BmECR   GLEQPSLVEEIQRYYLNTLRIYIINQNSASSRCAVIYGRILSVLTELRTL   559
MsECR   GLEQPLLVEEIQRYYLKTLRVYILNQHSASPRCAVLFGKILGVLTELRTL   501
HvECR   GLEQPLLVEDIQRYYLNTLRVYILNQNSASPRGAVIFGEILGILTEIRTL   518
CtECR   GLEKAEMVDIIQSYYTETLKVYIVRDHGGESRCSVQFAKLLGILTELRTM   484
AaECR   GLEQAELVEHIQSYYIDTLRIYILNRHAGDPKCSVIFAKLLSILTELRTL   542
DmECR   GLEKAQLVEAIQSYYIDTLRITILNRHCGDSMSLVFYAKLLSILTELRTL   624
        ***.. .*. **.** .**.. *.. ....   *  .. .*..***.**.
```

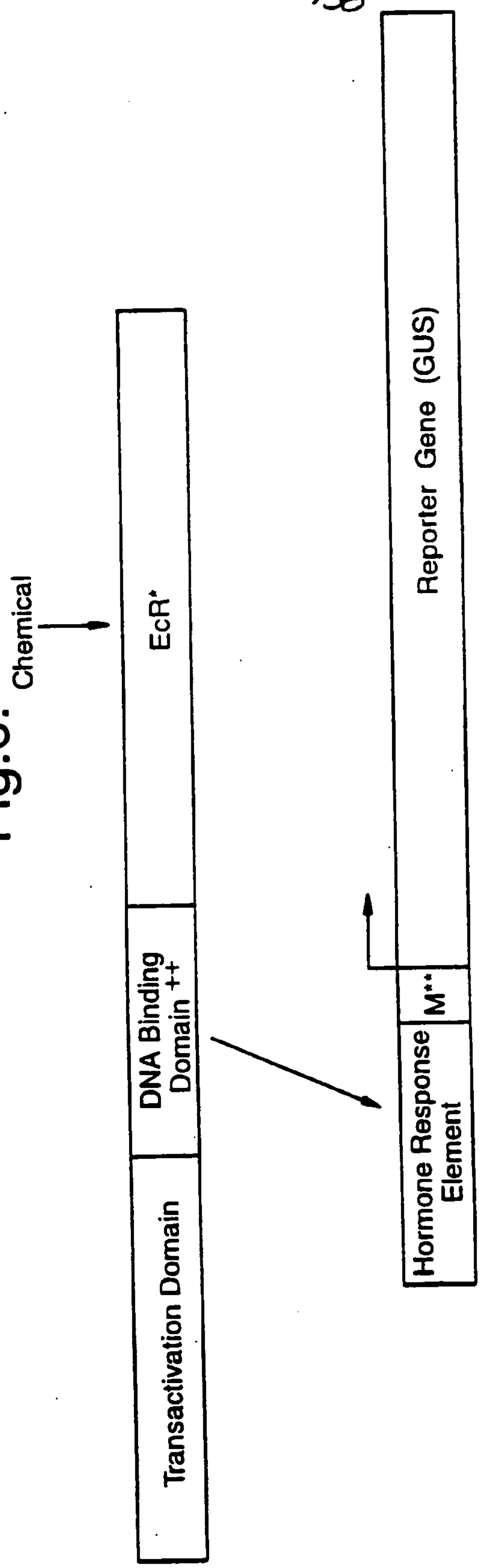
Fig.5 iv.

```
BmECR    GTQNSNMCISLKLKNRKLPPFLEEIWDVAEVARR----------------  593
MsECR    GTQNSNMCISLKLKNRKLPPFLEEIWDVAEVSTT----------------  535
HvECR    GMQNSNMCISLKLKKRKLPPFLEEIWDVADVATT----------------  552
CtECR    GNLNSEMCFSLKLRNRKLPRFLEEVWDVGDVNNQTTATTNTENIVRERIN  534
AaECR    GNQNSEMCFSLKLKNRKLPRFLEEIWDVQDIPPSMQAQMHSHGTQSSS--  590
DmECR    GNQNAEMCFSLKLKNRKLPKFLEEIWDVHAIPPSVQSHLQITQEEDERLE  674
         *  *..**.****..**** ****.***  ..

BmECR    --------------------------------------------------  593
MsECR    --------------------------------------------------  535
HvECR    --------------------------------------------------  552
CtECR    RN------------------------------------------------  536
AaECR    ------SSSSSSSSSSNGSSNGNSSSNSNSSQHGPHPHPHGQQ--LTPNQ  632
DmECR    RAERMRASVGGAITAGIDCDSASTSAAAAAAQHQPQPQPQPQPSSLTQND  724

BmECR    ---------------------HPTV----------LPPTNPVVL------  606
MsECR    ---------------------QP--TPGVAAQVTPIVVDNPAAL------  556
HvECR    ---------------------ATPVAAEAPAPLAPAPPARPATV------  575
CtECR    --------------------------------------------------  536
AaECR    QQHQQQ-----------HSQLQQ--------------------------V  645
DmECR    SQHQTQPQLQPQLPPQLQGQLQPQLQPQLQTQLQPQIQPQPQLLPVSAPV  774

BmECR    --------------------------------------------------  606
MsECR    --------------------------------------------------  556
HvECR    --------------------------------------------------  575
CtECR    --------------------------------------------------  536
AaECR    HANGSGSGGGSNNNSSSG--------------------------------  663
DmECR    PASVTAPGSLSAVSTSSEYMGGSAAIGPITPATTSSITAAVTASSTTSAV  824
```

Fig.5 v.

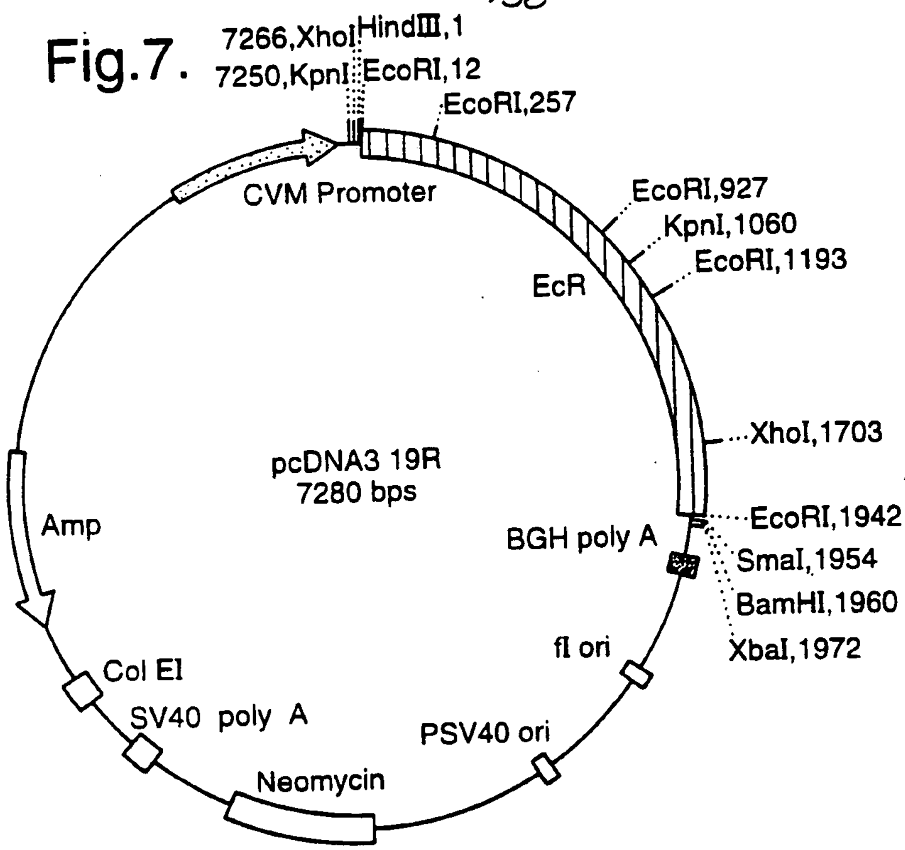
```
BmECR    --------------------------------------------------   606
MsECR    --------------------------------------------------   556
HvECR    --------------------------------------------------   575
CtECR    --------------------------------------------------   536
AaECR    ----GVVPGLGMLDQV----------------------------------   675
DmECR    PMGNGVGVGVGVGGNVSMYANAQTAMALMGVALHSHQQQLIGGVAVKSEH   874

BmECR    ----   606
MsECR    ----   556
HvECR    ----   575
CtECR    ----   536
AaECR    ----   675
DmECR    STTA   878
```

Fig.6.



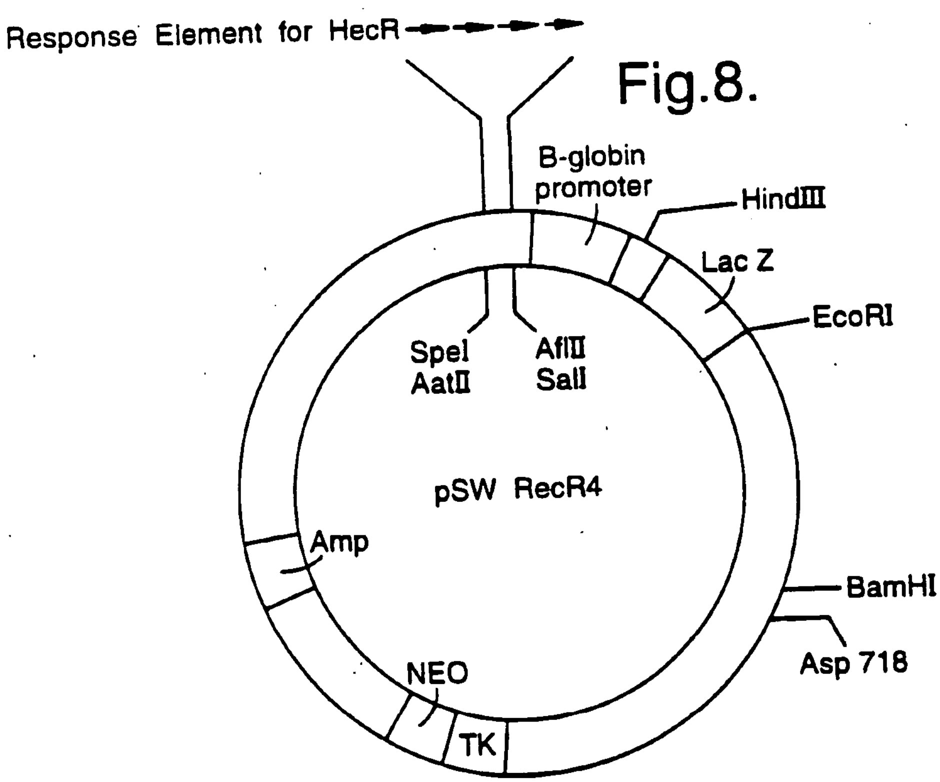
++ Glucocorticoid receptor DNA binding and transactivation domains
* Insect ecdysone ligand binding domain
** Minimal 35S CaMV promoter

Fig.7.



7266,XhoI
HindIII,1
7250,KpnI
EcoRI,12
EcoRI,257
CVM Promoter
EcoRI,927
KpnI,1060
EcoRI,1193
EcR
XhoI,1703
pcDNA3 19R
7280 bps
Amp
BGH poly A
EcoRI,1942
SmaI,1954
BamHI,1960
XbaI,1972
fl ori
Col EI
SV40 poly A
PSV40 ori
Neomycin

Fig.8.



Response Element for HecR
B-globin promoter
HindIII
Lac Z
EcoRI
SpeI
AatII
AflII
SalI
pSW RecR4
Amp
BamHI
Asp 718
NEO
TK

Fig.9.

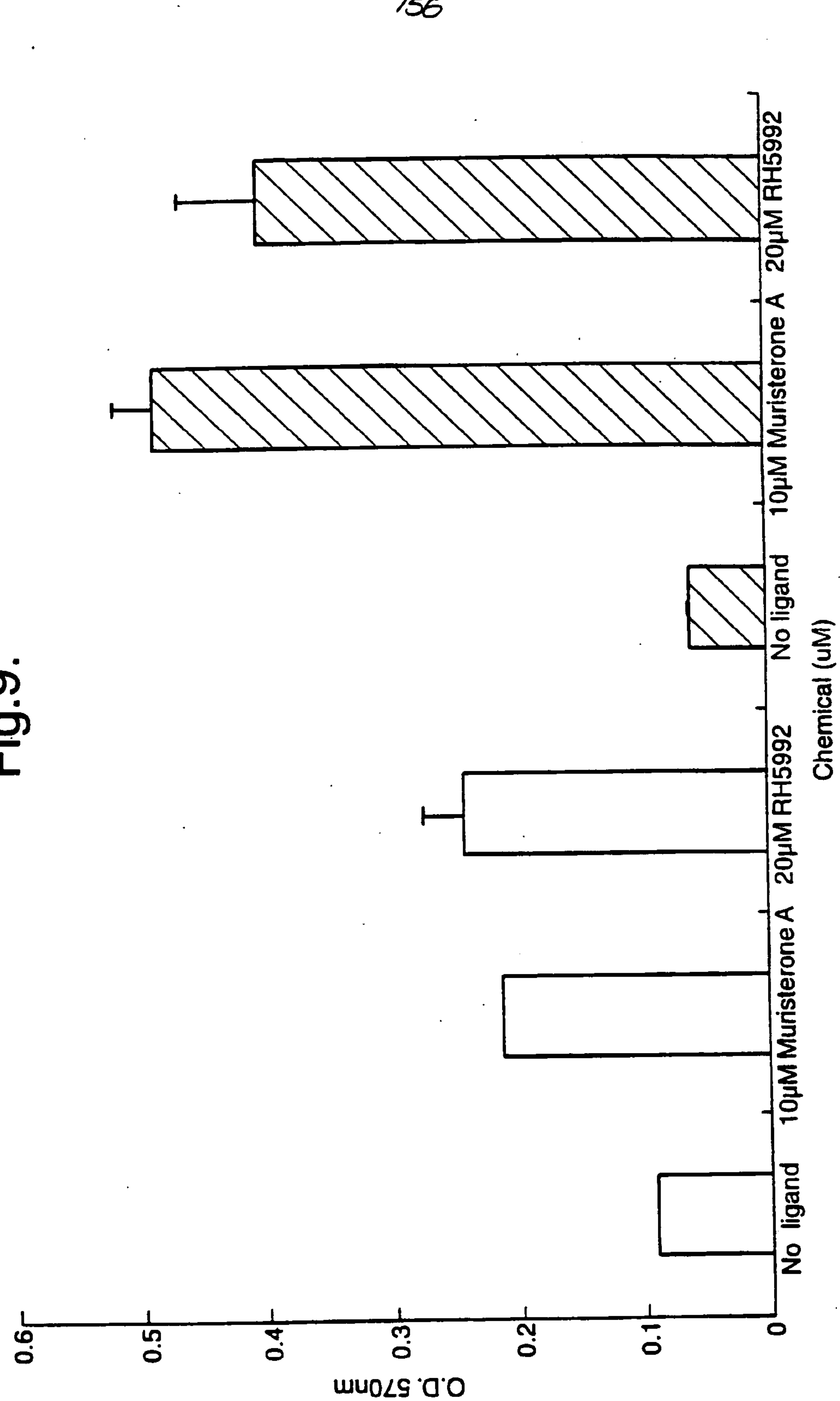

O.D. 570nm
0
0.1
0.2
0.3
0.4
0.5
0.6
No ligand
10µM Muristerone A
20µM RH5992
No ligand
10µM Muristerone A
20µM RH5992
Chemical (uM)

Fig.10.

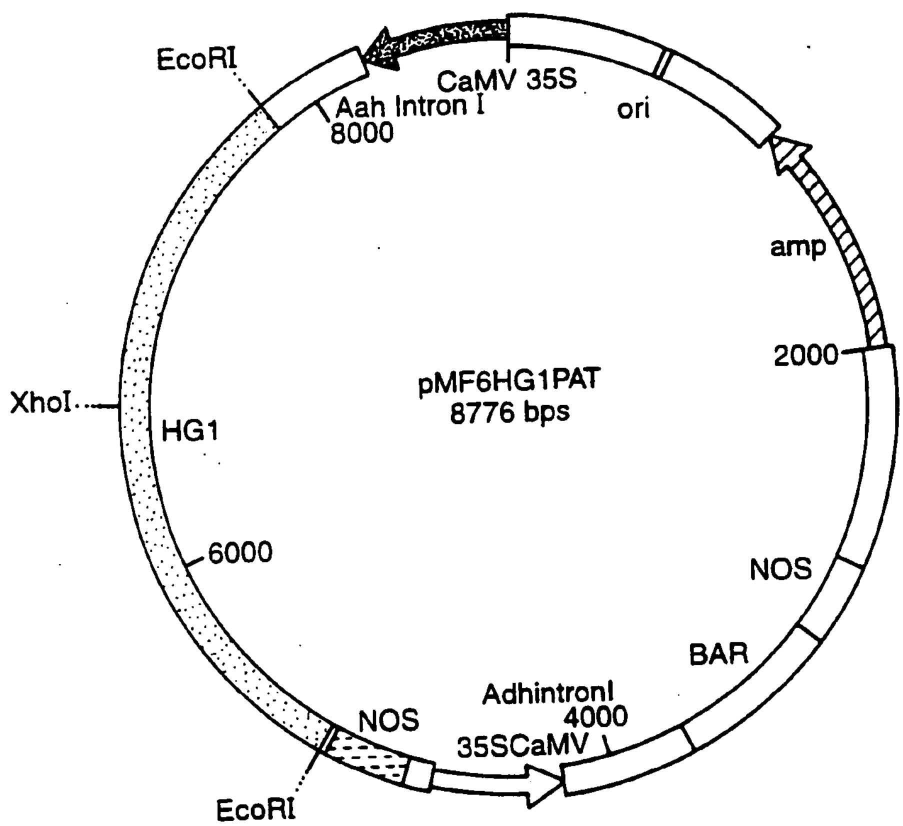

EcoRI
CaMV 35S
ori
Aah Intron I
8000
amp
2000
pMF6HG1PAT
8776 bps
XhoI
HG1
6000
NOS
BAR
AdhintronI
4000
NOS
35SCaMV
EcoRI

Fig.11.

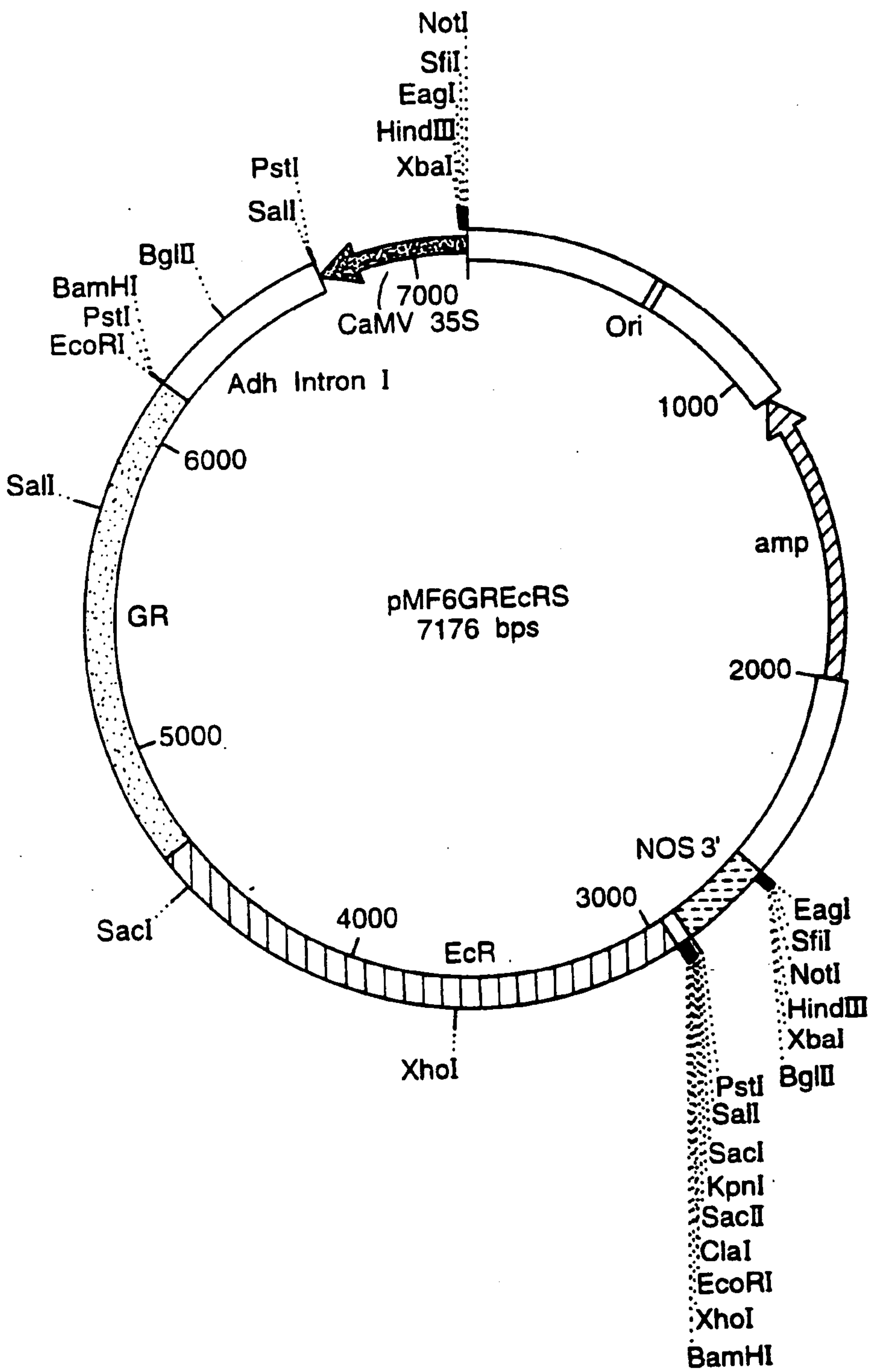

NotI
SfiI
EagI
HindIII
XbaI
PstI
SalI
BglII
BamHI
PstI
EcoRI
7000
CaMV 35S
Ori
Adh Intron I
1000
6000
SalI
amp
GR
pMF6GREcRS
7176 bps
2000
5000
NOS 3'
3000
EagI
SfiI
NotI
HindIII
XbaI
BglII
SacI
4000
EcR
XhoI
PstI
SalI
SacI
KpnI
SacII
ClaI
EcoRI
XhoI
BamHI

Fig.12.

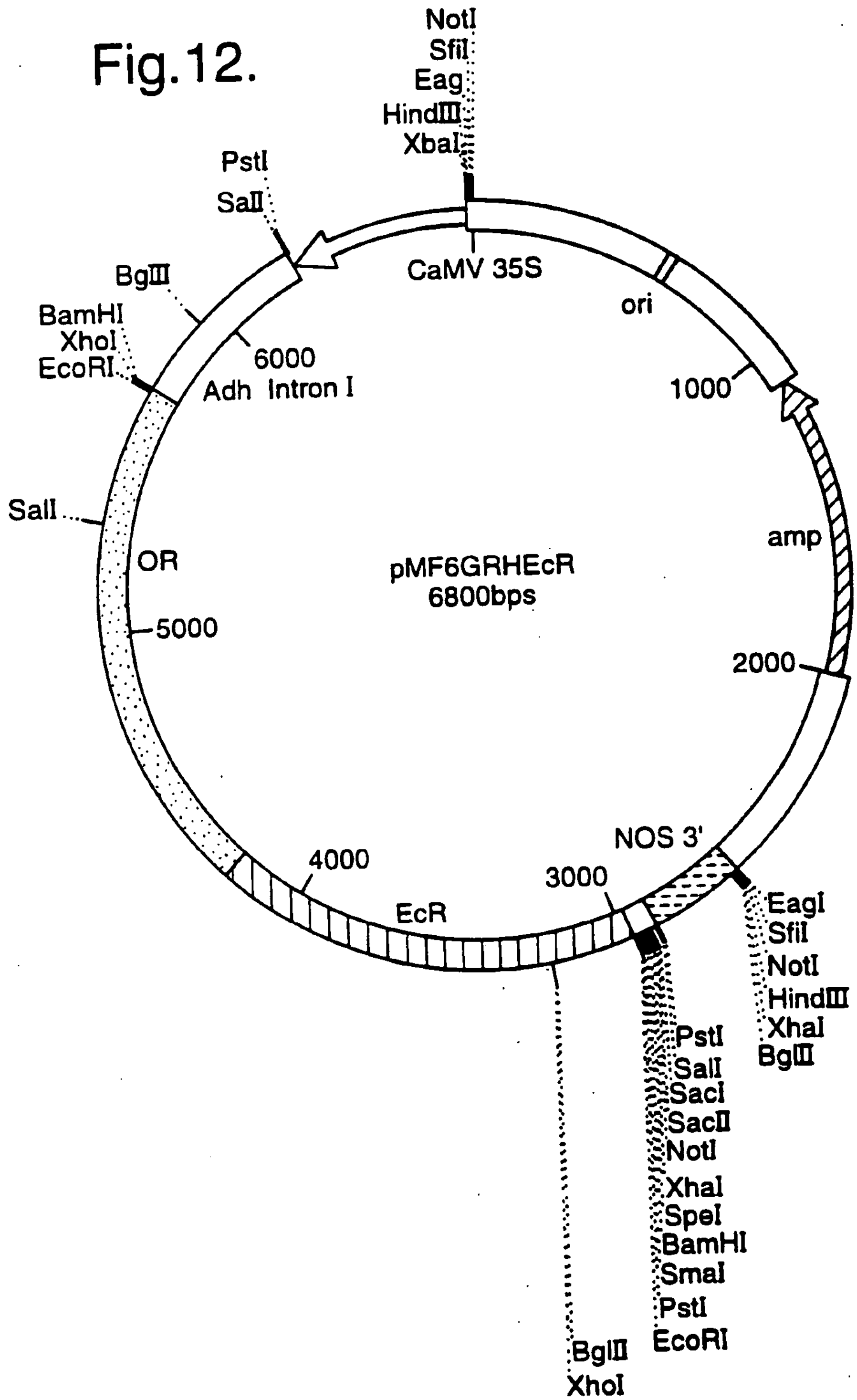

NotI
SfiI
Eag
HindIII
XbaI
PstI
SalI
BglII
BamHI
XhoI
EcoRI
CaMV 35S
ori
6000
Adh Intron I
1000
SalI
amp
OR
pMF6GRHEcR
6800bps
5000
2000
NOS 3'
4000
3000
EagI
SfiI
NotI
HindIII
XhaI
BglII
EcR
PstI
SalI
SacI
SacII
NotI
XhaI
SpeI
BamHI
SmaI
PstI
EcoRI
BglII
XhoI

Fig.13.

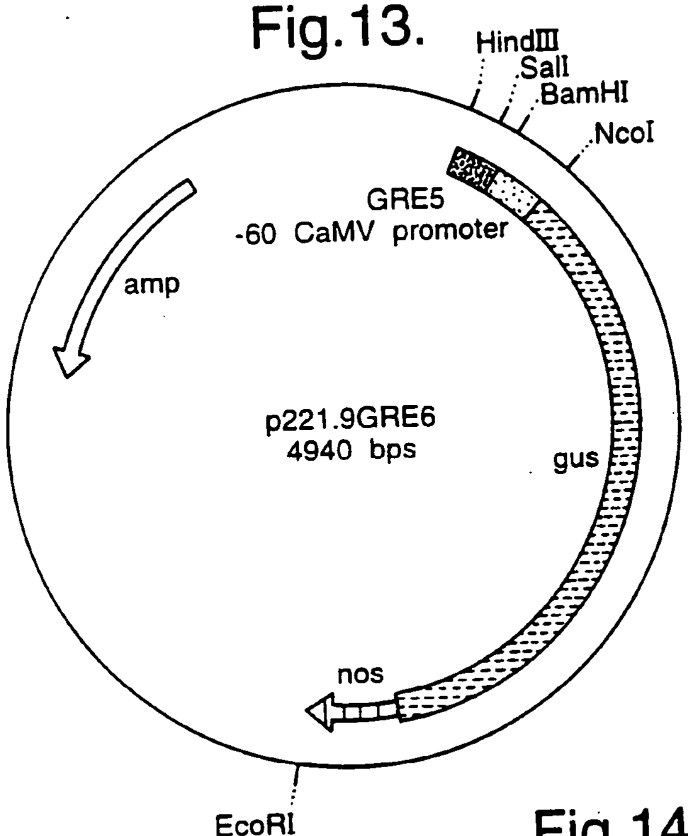

HindIII
SalI
BamHI
NcoI
GRE5
-60 CaMV promoter
amp
p221.9GRE6
4940 bps
gus
nos
EcoRI

Fig.14.

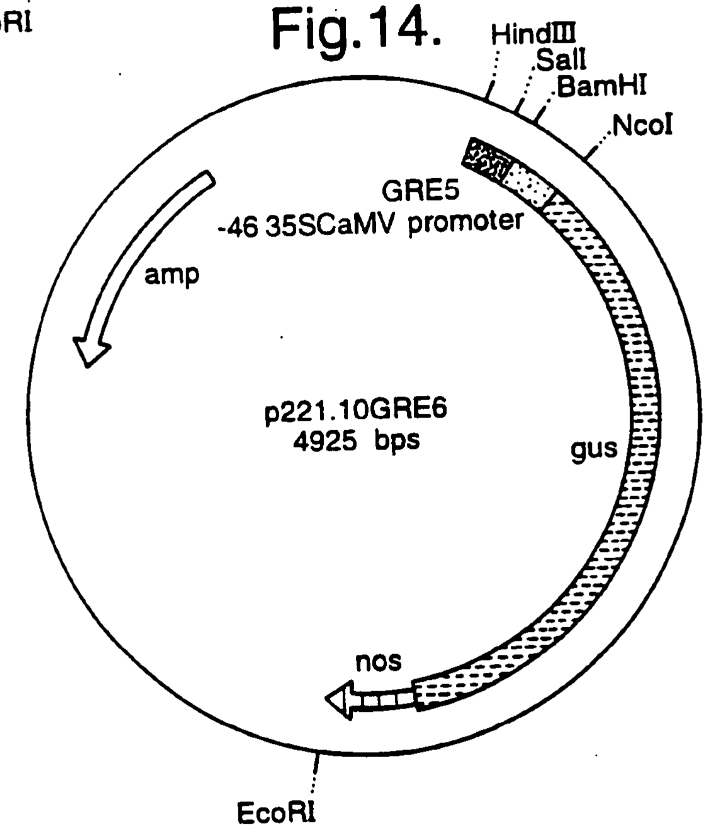

HindIII
SalI
BamHI
NcoI
GRE5
-46 35SCaMV promoter
amp
p221.10GRE6
4925 bps
gus
nos
EcoRI

Fig.15.

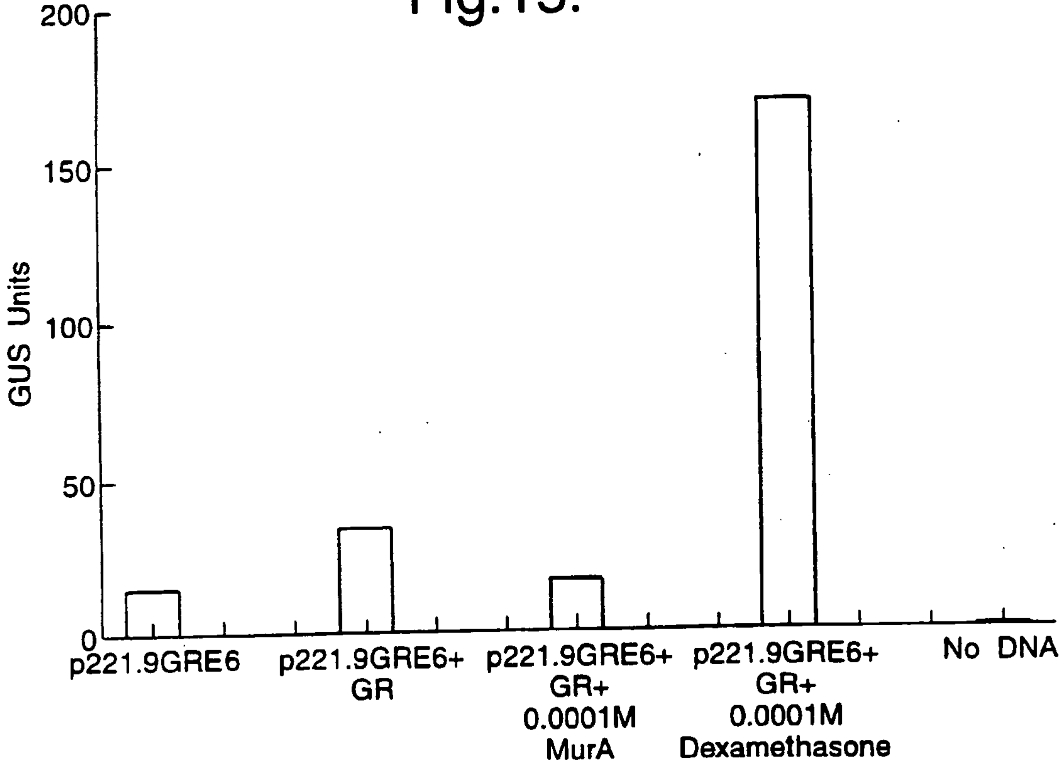

200
150
100
50
0
GUS Units
p221.9GRE6
p221.9GRE6+ GR
p221.9GRE6+ GR+ 0.0001M MurA
p221.9GRE6+ GR+ 0.0001M Dexamethasone
No DNA

Fig.16.

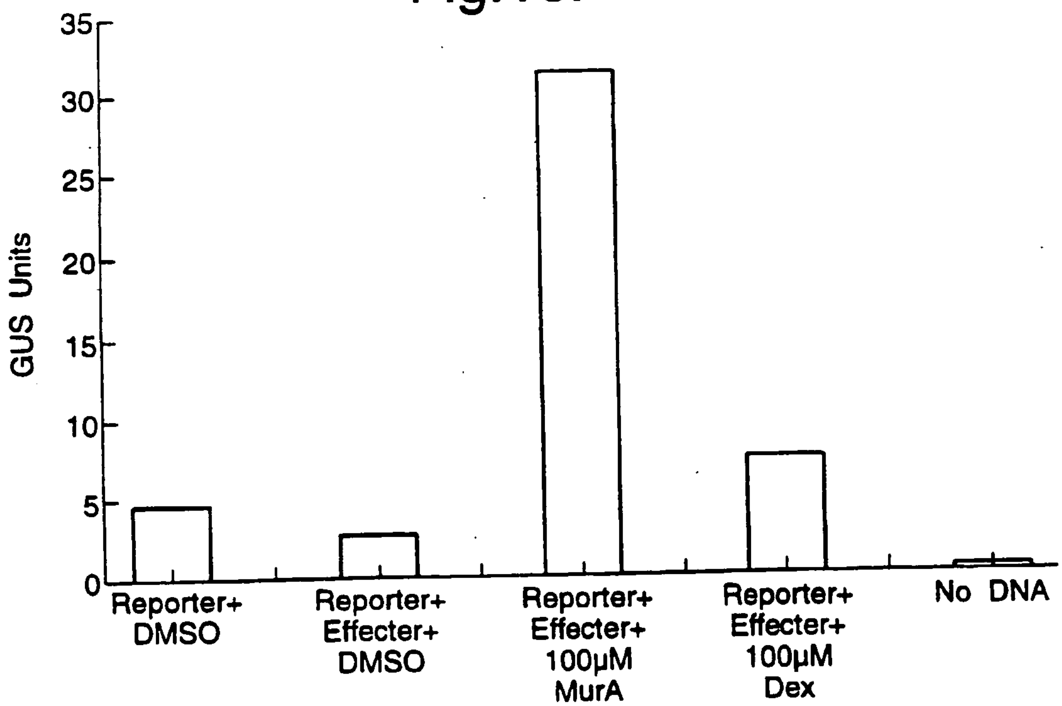

35
30
25
20
15
10
5
0
GUS Units
Reporter+ DMSO
Reporter+ Effecter+ DMSO
Reporter+ Effecter+ 100μM MurA
Reporter+ Effecter+ 100μM Dex
No DNA

Fig.17.

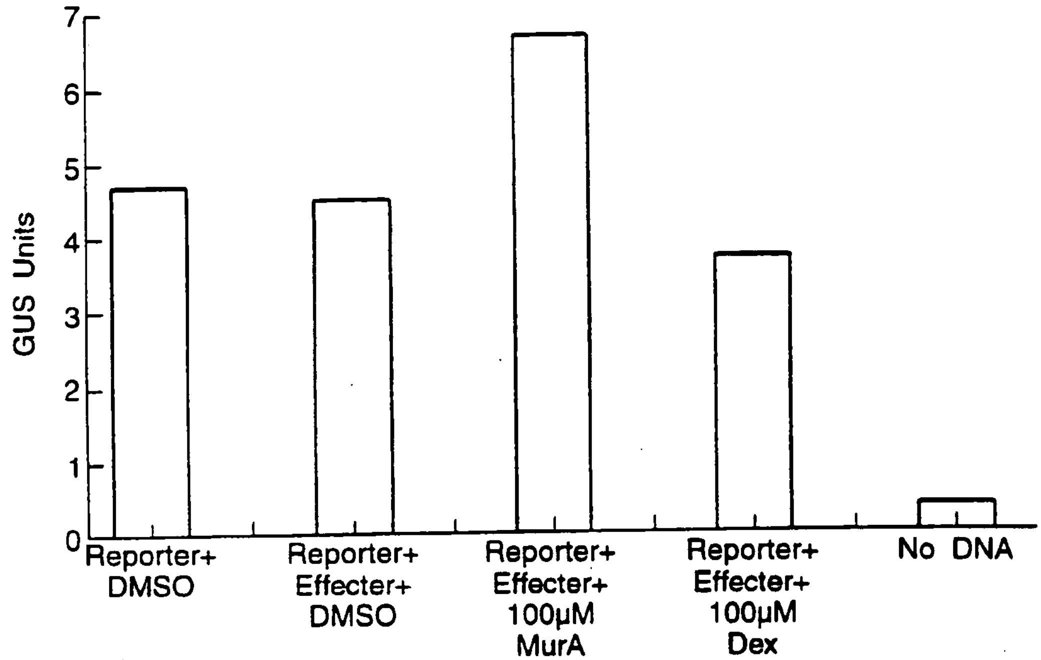

GUS Units
0
1
2
3
4
5
6
7
Reporter+ DMSO
Reporter+ Effecter+ DMSO
Reporter+ Effecter+ 100μM MurA
Reporter+ Effecter+ 100μM Dex
No DNA

Fig.18.

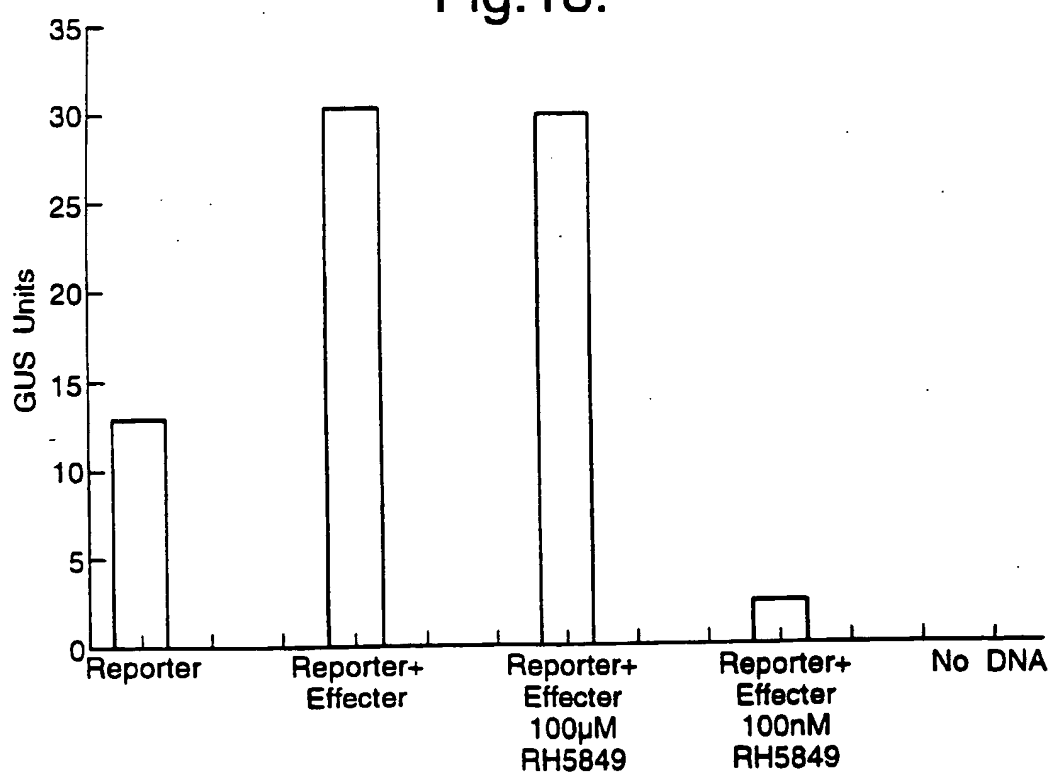

GUS Units
0
5
10
15
20
25
30
35
Reporter
Reporter+ Effecter
Reporter+ Effecter 100μM RH5849
Reporter+ Effecter 100nM RH5849
No DNA

Fig.19.

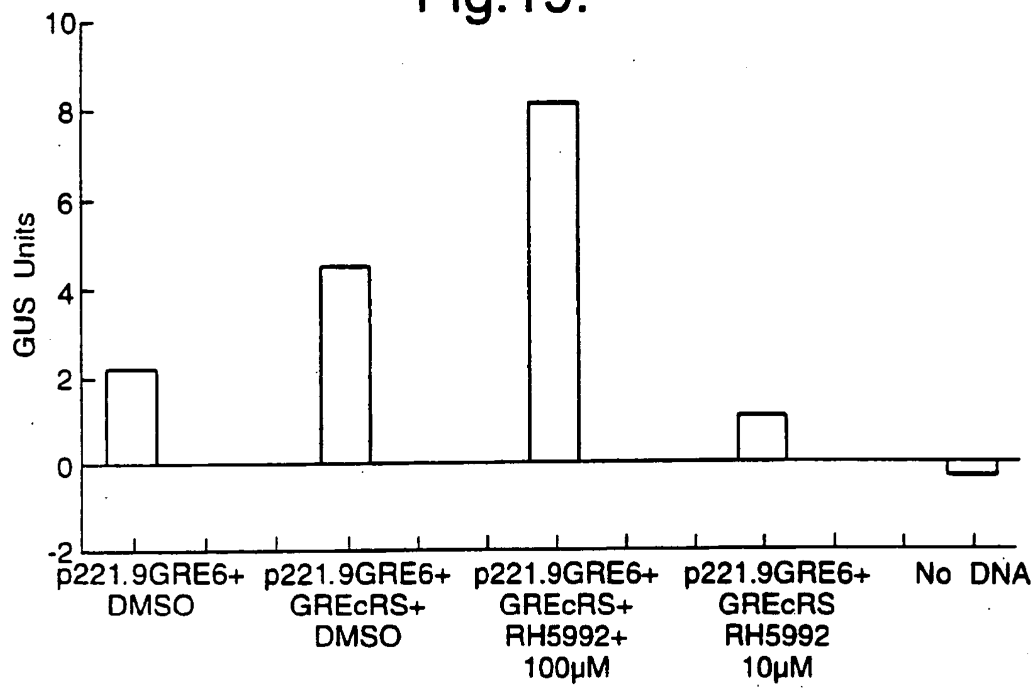

10
8
6
4
2
0
-2
GUS Units
p221.9GRE6+
DMSO
p221.9GRE6+
GREcRS+
DMSO
p221.9GRE6+
GREcRS+
RH5992+
100μM
p221.9GRE6+
GREcRS
RH5992
10μM
No DNA

Fig.20.

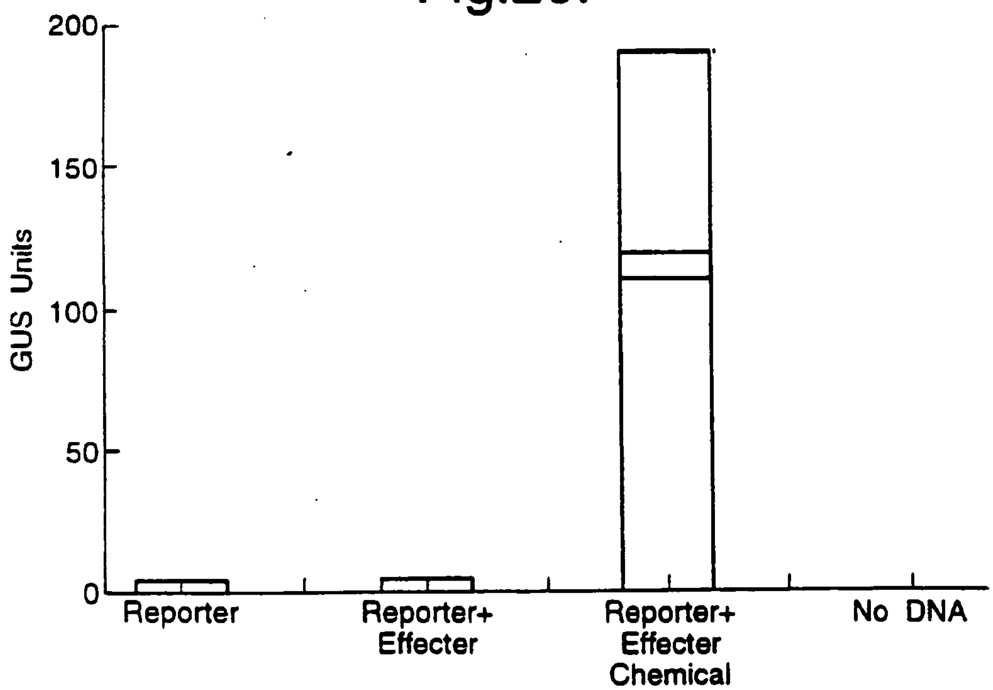

200
150
100
50
0
GUS Units
Reporter
Reporter+
Effecter
Reporter+
Effecter
Chemical
No DNA

Fig.21.

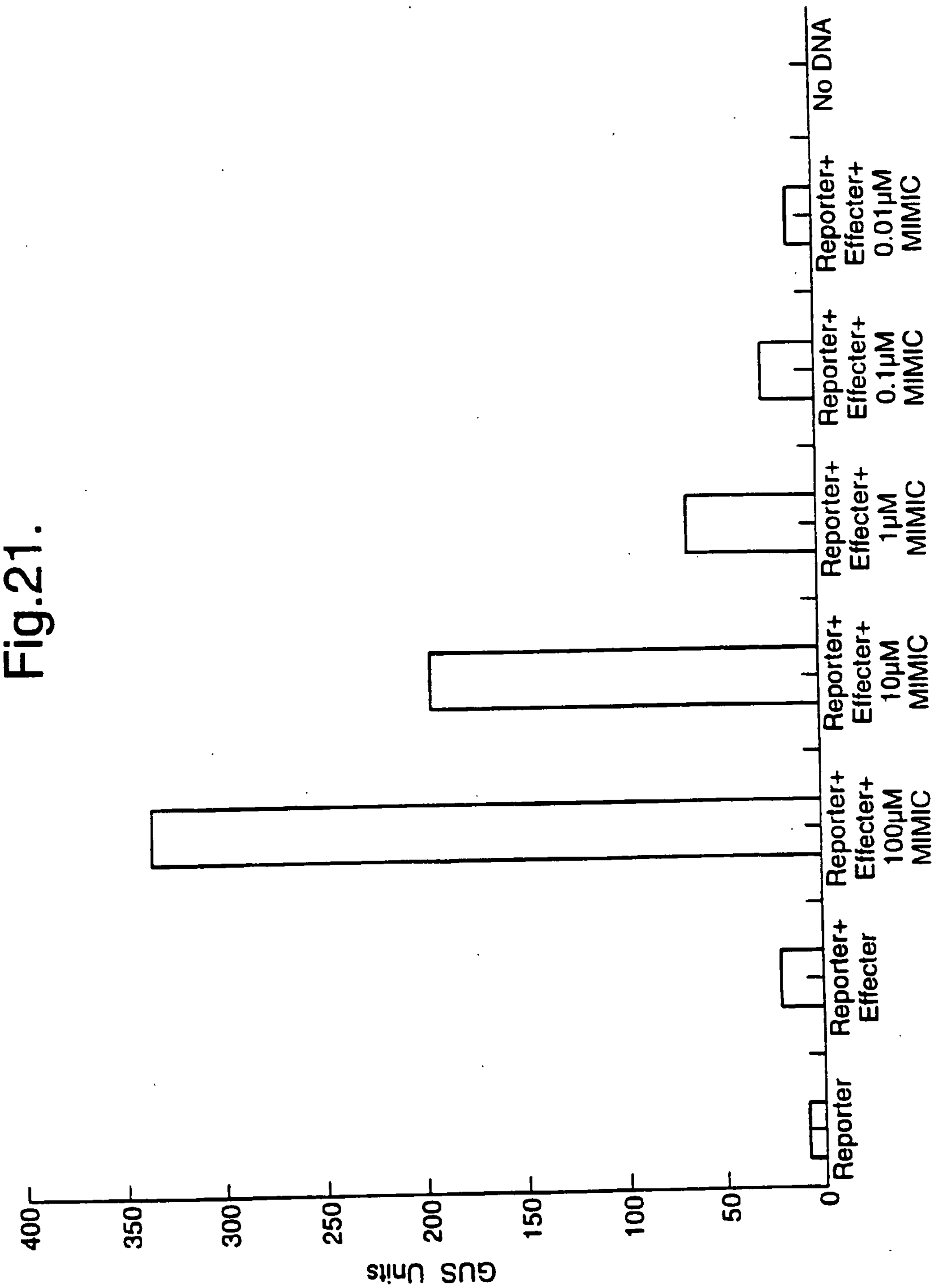

GUS Units
0
50
100
150
200
250
300
350
400
Reporter
Reporter+ Effecter
Reporter+ Effecter+ 100μM MIMIC
Reporter+ Effecter+ 10μM MIMIC
Reporter+ Effecter+ 1μM MIMIC
Reporter+ Effecter+ 0.1μM MIMIC
Reporter+ Effecter+ 0.01μM MIMIC
No DNA

Fig.22.

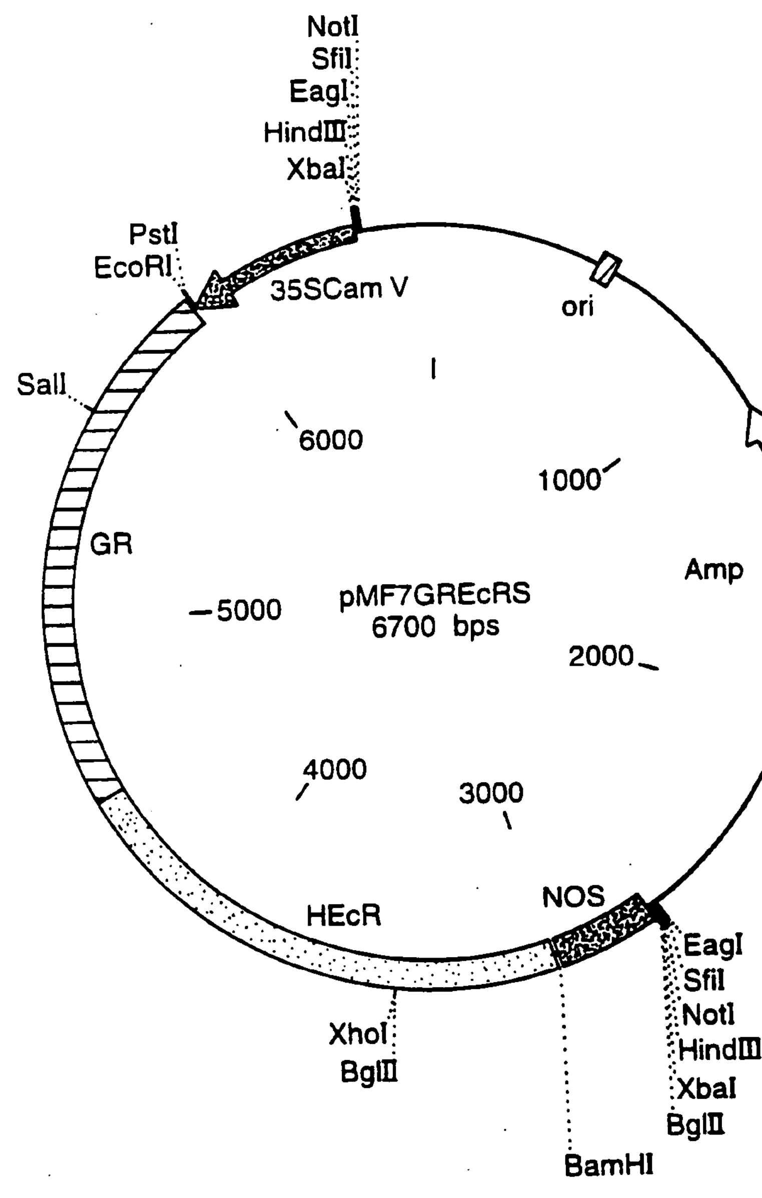

NotI
SfiI
EagI
HindIII
XbaI
PstI
EcoRI
35SCam V
ori
SalI
6000
1000
GR
Amp R
5000
pMF7GREcRS
6700 bps
2000
4000
3000
NOS
HEcR
EagI
SfiI
NotI
HindIII
XbaI
BglII
XhoI
BglII
BamHI

Fig.23.

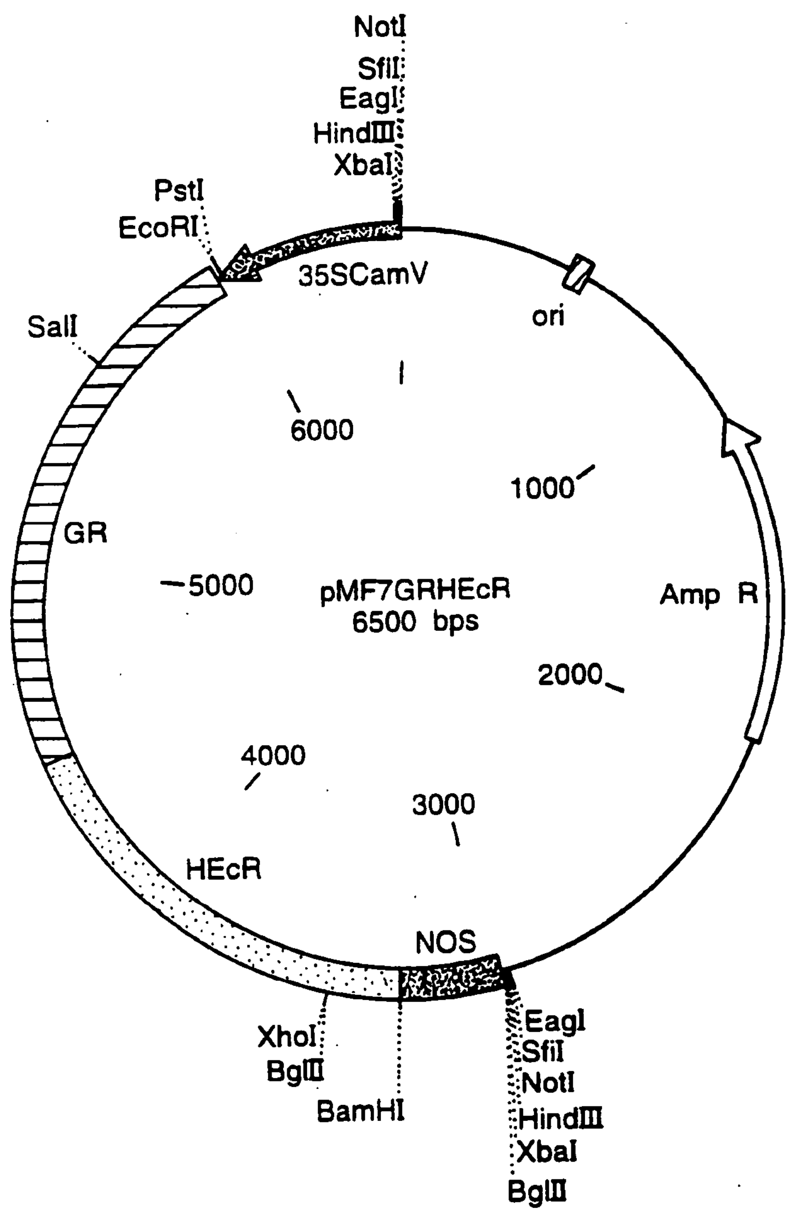
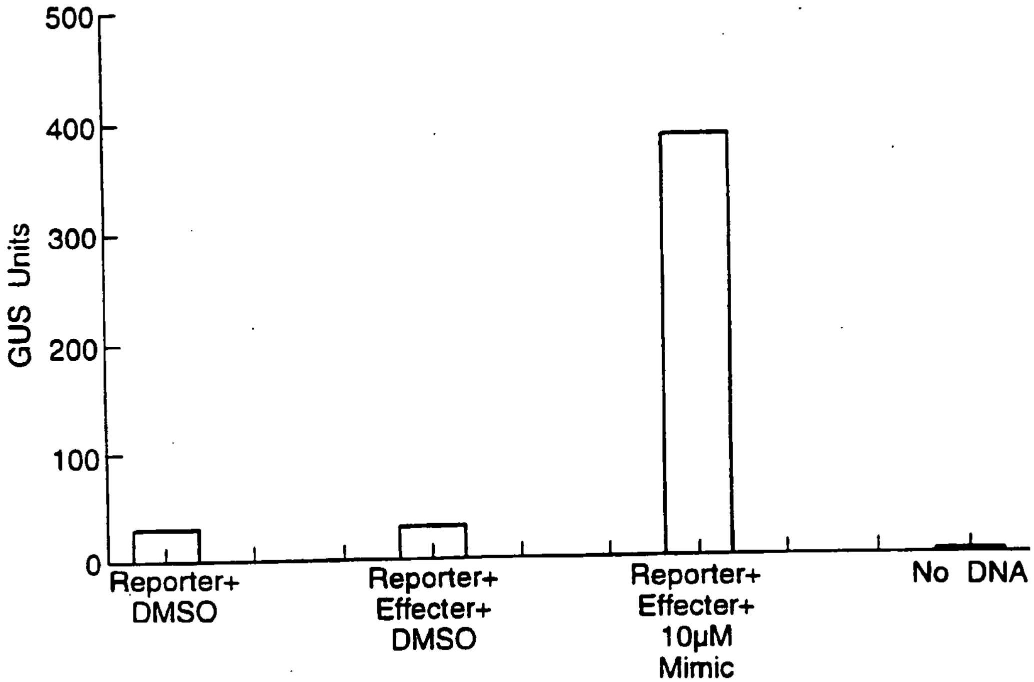
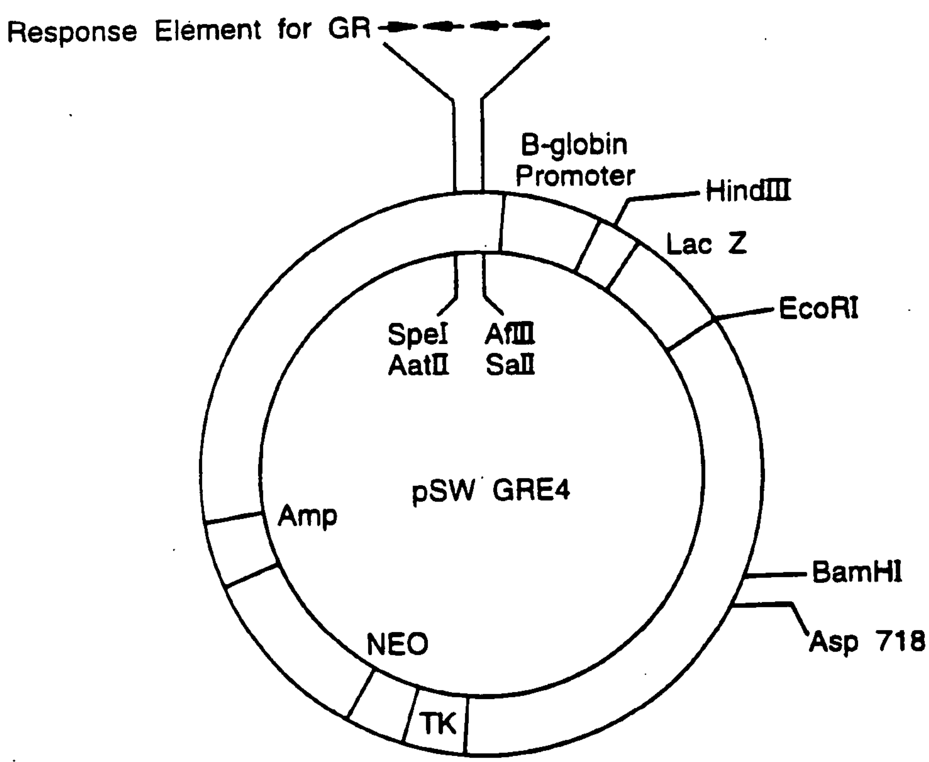

NotI
SfiI
EagI
HindIII
XbaI
PstI
EcoRI
35SCamV
ori
SalI
6000
1000
GR
5000
pMF7GRHEcR
6500 bps
Amp R
2000
4000
3000
HEcR
NOS
XhoI
BglII
BamHI
EagI
SfiI
NotI
HindIII
XbaI
BglII

Fig.24.



GUS Units
500
400
300
200
100
0
Reporter+ DMSO
Reporter+ Effecter+ DMSO
Reporter+ Effecter+ 10μM Mimic
No DNA

Fig.26.



Response Element for GR
B-globin Promoter
HindIII
Lac Z
EcoRI
SpeI AatII
AflII SalI
pSW GRE4
Amp
BamHI
Asp 718
NEO
TK

Fig.25.

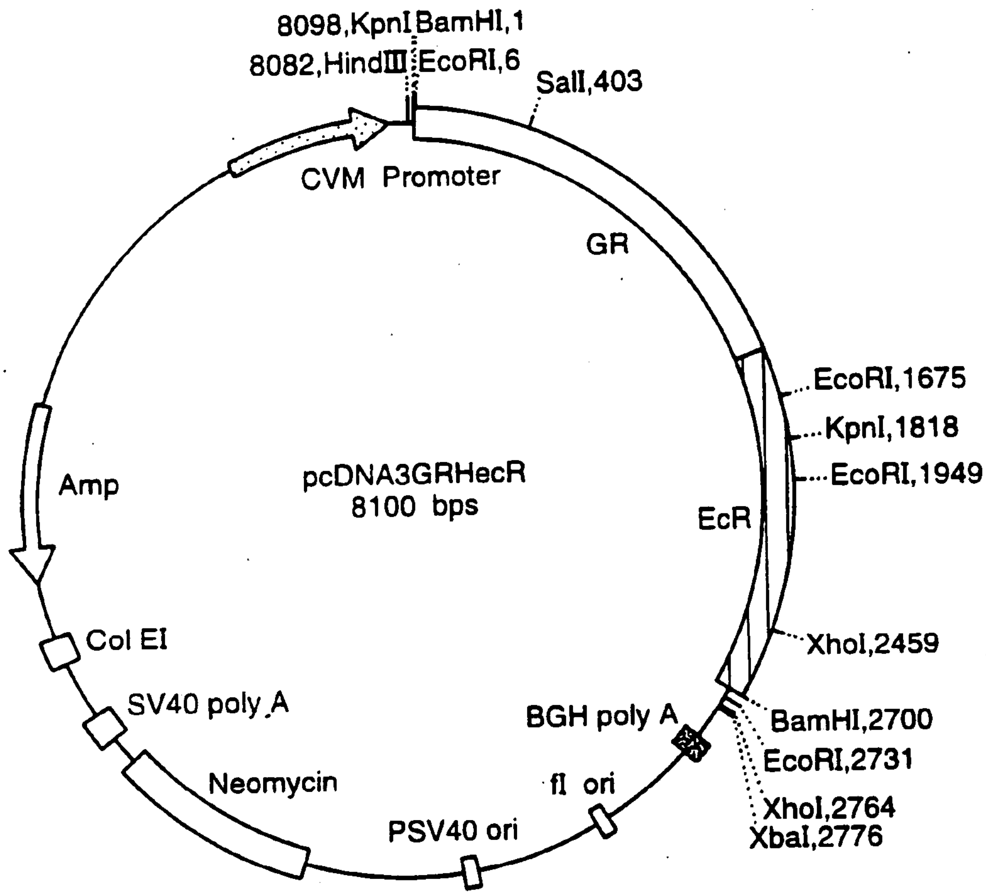

8098,KpnI BamHI,1
8082,HindIII EcoRI,6
SalI,403
CVM Promoter
GR
EcoRI,1675
KpnI,1818
EcoRI,1949
EcR
XhoI,2459
BamHI,2700
EcoRI,2731
XhoI,2764
XbaI,2776
BGH poly A
fI ori
PSV40 ori
Neomycin
SV40 poly A
Col EI
Amp
pcDNA3GRHecR
8100 bps

Fig.27.

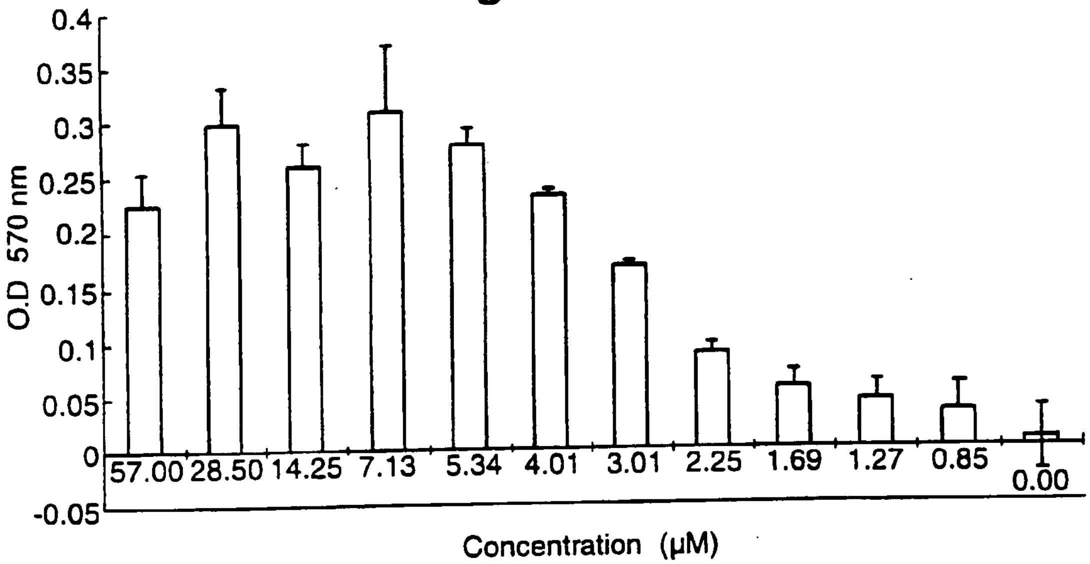

0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
-0.05
O.D 570 nm
57.00
28.50
14.25
7.13
5.34
4.01
3.01
2.25
1.69
1.27
0.85
0.00
Concentration (µM)

Fig.28.

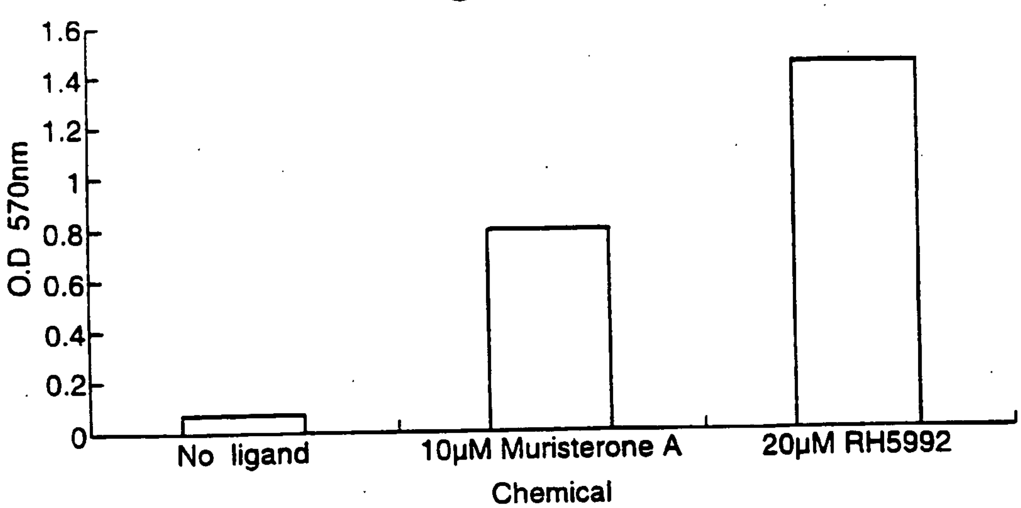

1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
O.D 570nm
No ligand
10µM Muristerone A
20µM RH5992
Chemical

Fig.29.

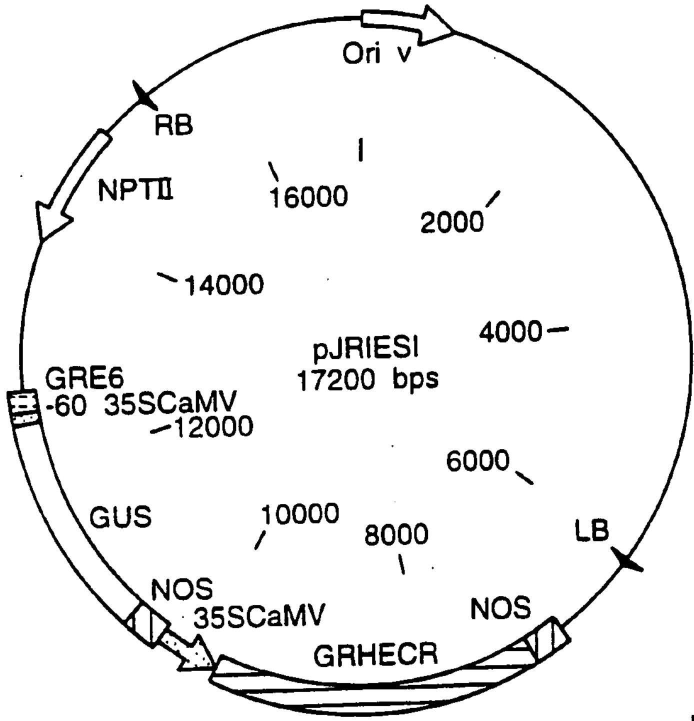

Ori v
RB
NPTII
16000
2000
14000
4000
pJRIESI
17200 bps
GRE6
-60 35SCaMV
12000
6000
GUS
10000
8000
LB
NOS
35SCaMV
NOS
GRHECR

Fig.30.

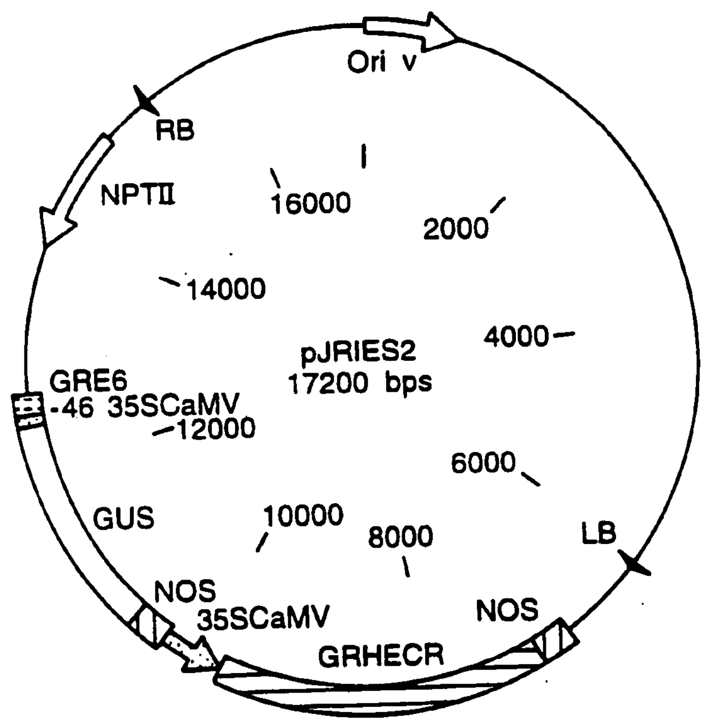

Ori v
RB
NPTII
16000
2000
14000
4000
pJRIES2
17200 bps
GRE6
-46 35SCaMV
12000
6000
GUS
10000
8000
LB
NOS
35SCaMV
NOS
GRHECR

Fig.31.

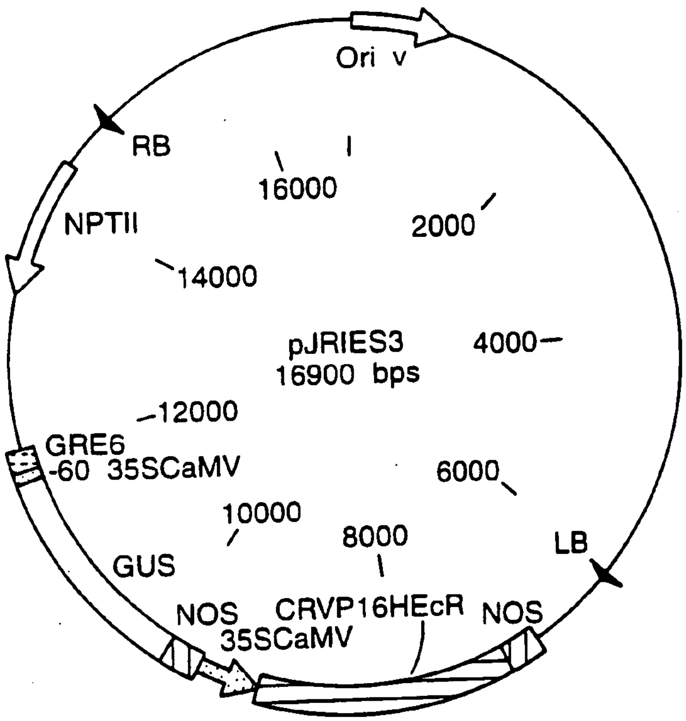

Ori v
RB
16000
2000
NPTII
14000
pJRIES3
16900 bps
4000
12000
GRE6
-60 35SCaMV
6000
10000
8000
LB
GUS
NOS
35SCaMV
CRVP16HEcR
NOS

Fig.32.

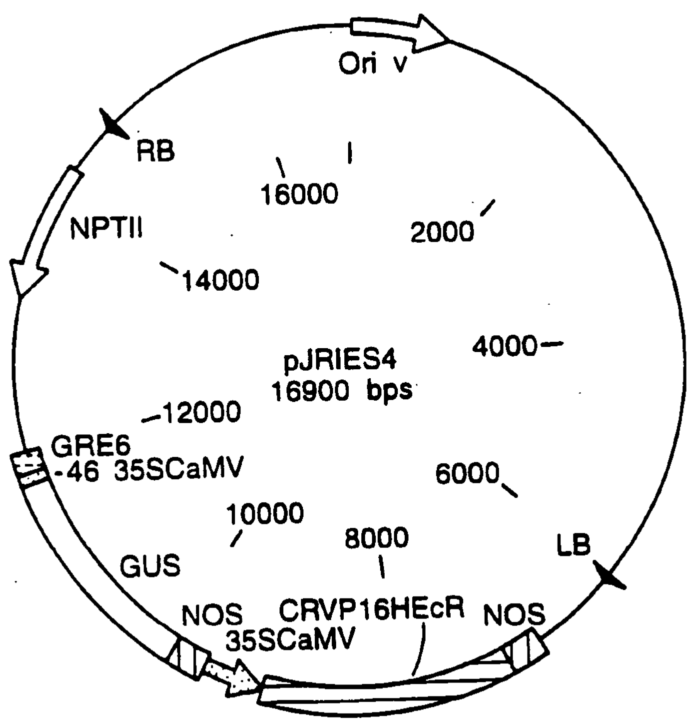

Ori v
RB
16000
2000
NPTII
14000
pJRIES4
16900 bps
4000
12000
GRE6
-46 35SCaMV
6000
10000
8000
LB
GUS
NOS
35SCaMV
CRVP16HEcR
NOS

Fig.33.

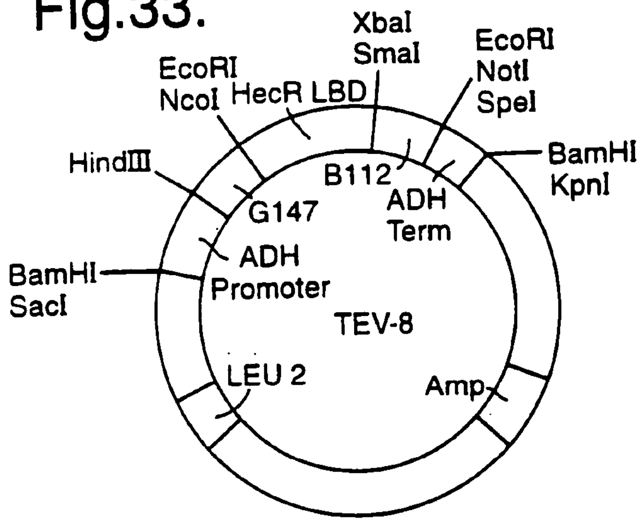


Fig.34.

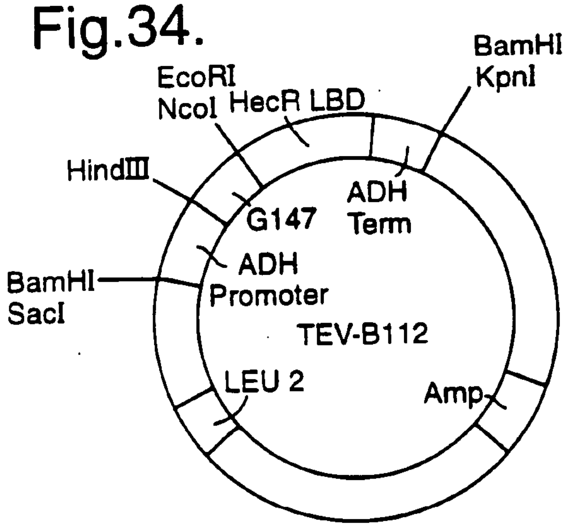


Fig.35.

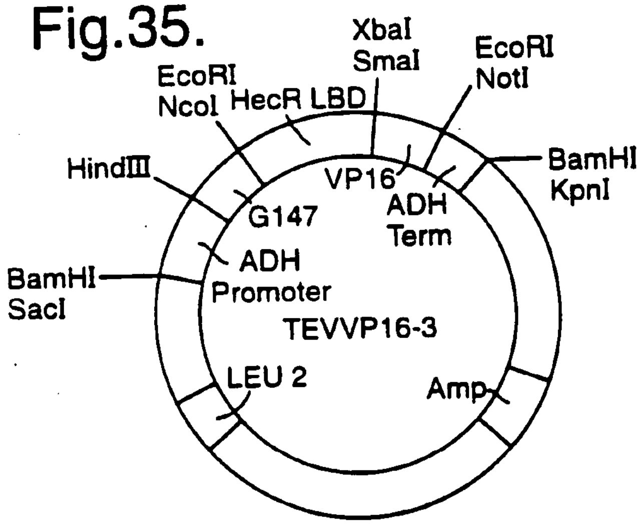


Fig.36.

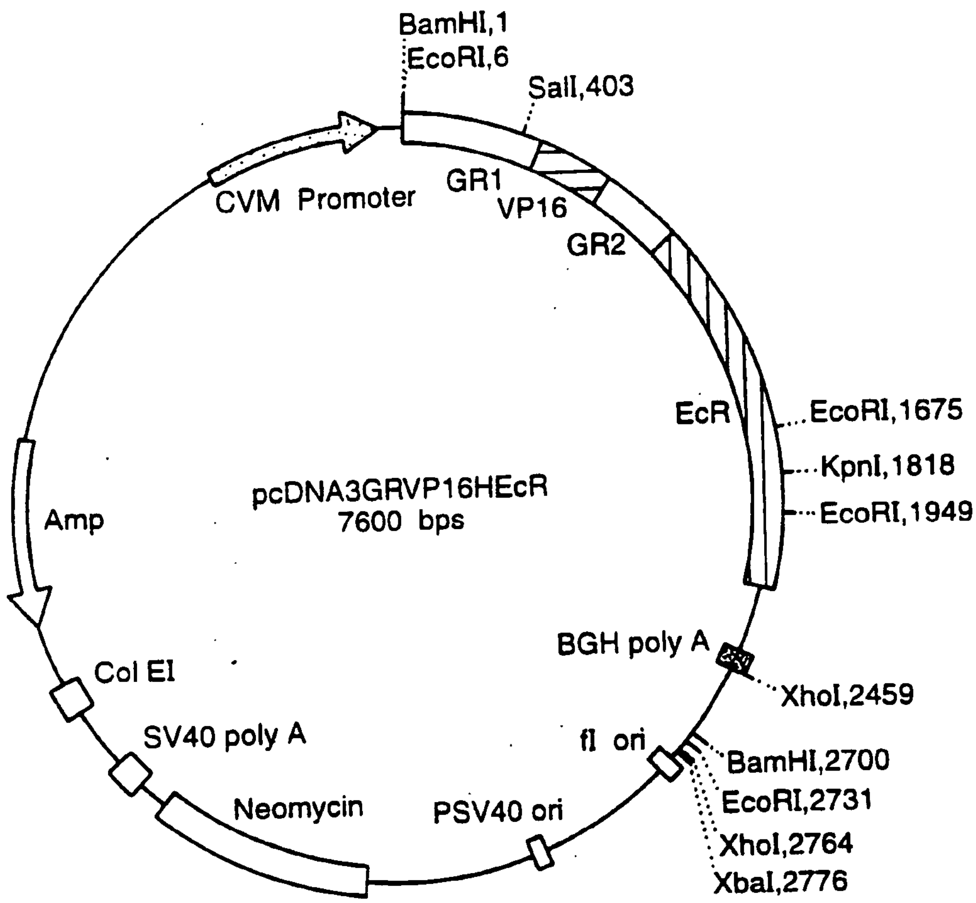

BamHI,1
EcoRI,6
SalI,403
GR1
VP16
GR2
CVM Promoter
EcR
EcoRI,1675
KpnI,1818
EcoRI,1949
Amp
pcDNA3GRVP16HEcR
7600 bps
BGH poly A
XhoI,2459
Col EI
SV40 poly A
fI ori
BamHI,2700
EcoRI,2731
XhoI,2764
XbaI,2776
Neomycin
PSV40 ori

Fig.37.

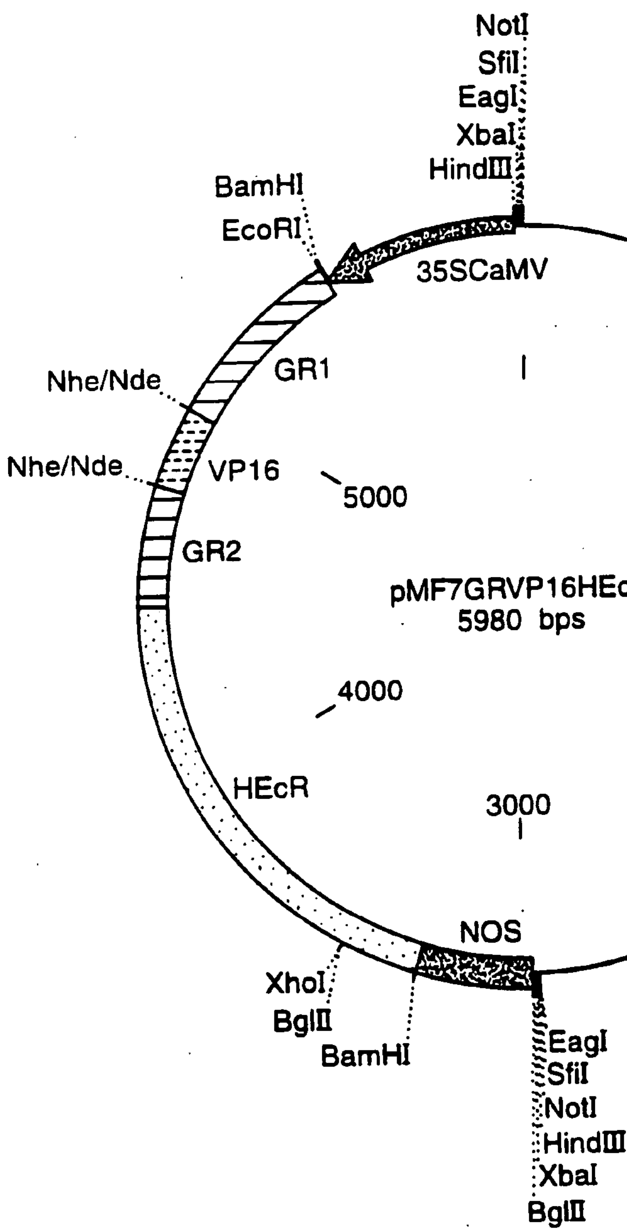

NotI
SfiI
EagI
XbaI
HindIII
BamHI
EcoRI
35SCaMV
Ori
GR1
Nhe/Nde
Nhe/Nde
VP16
5000
1000
GR2
pMF7GRVP16HEcR
5980 bps
Amp R
4000
2000
HEcR
3000
NOS
XhoI
BglII
BamHI
EagI
SfiI
NotI
HindIII
XbaI
BglII

Fig.38.

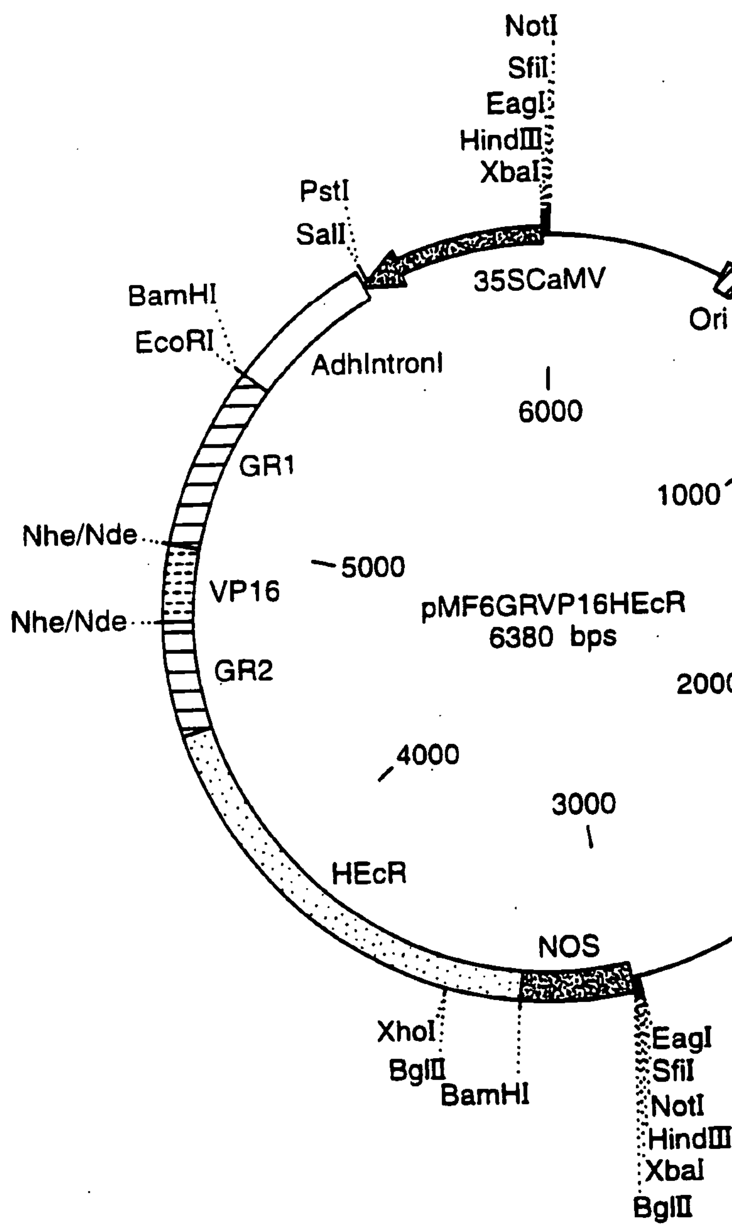
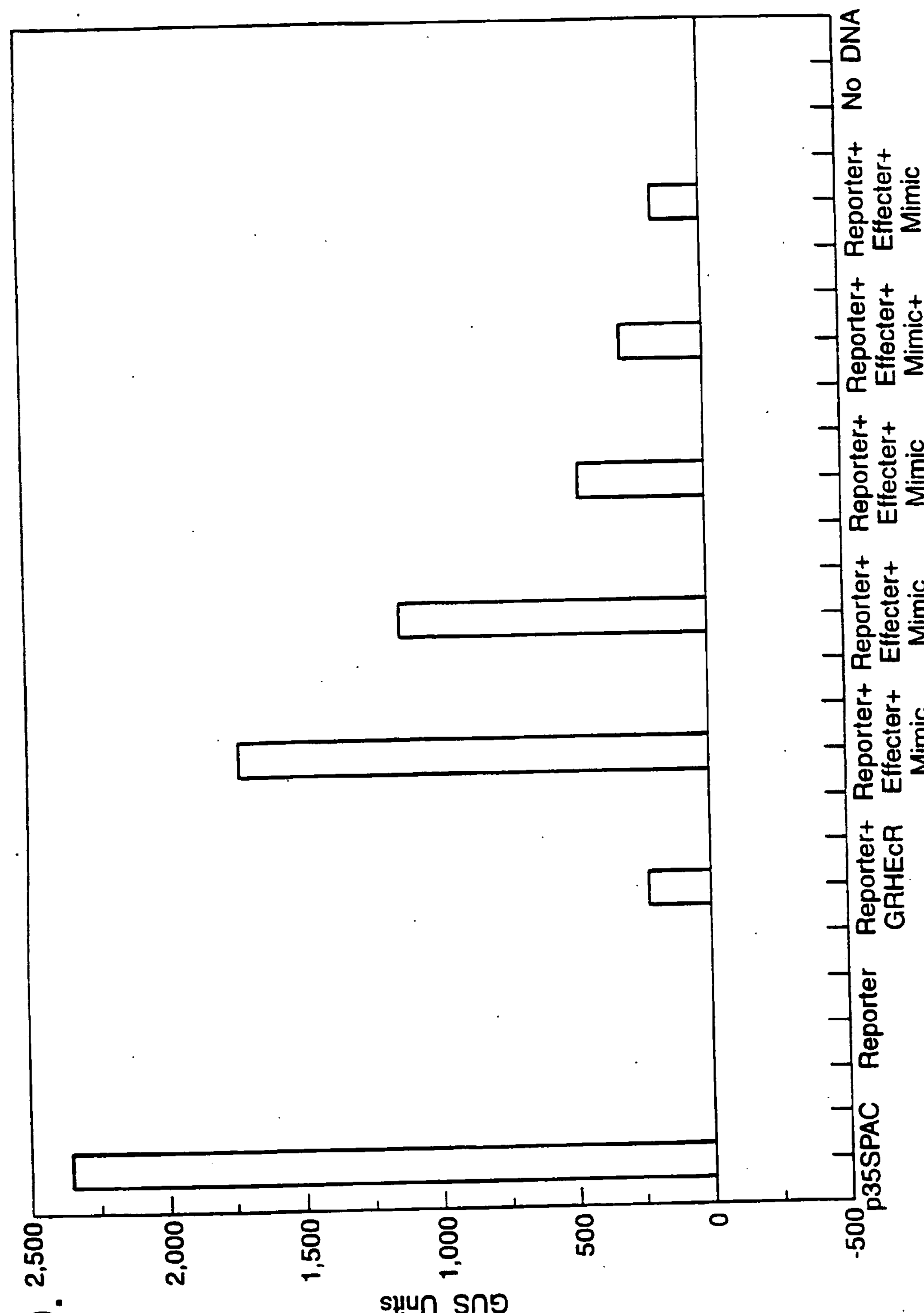

NotI
SfiI
EagI
HindIII
XbaI
PstI
SalI
35SCaMV
Ori
BamHI
EcoRI
AdhIntronI
6000
GR1
1000
Nhe/Nde
5000
VP16
pMF6GRVP16HEcR
6380 bps
Amp R
Nhe/Nde
GR2
2000
4000
3000
HEcR
NOS
XhoI
BglII
BamHI
EagI
SfiI
NotI
HindIII
XbaI
BglII

Fig.39.


GUS Units
2,500
2,000
1,500
1,000
500
0
-500
p35SPAC
Reporter
Reporter+ GRHEcR
Reporter+ Effecter+ Mimic 100µM
Reporter+ Effecter+ Mimic 10uM
Reporter+ Effecter+ Mimic 1uM
Reporter+ Effecter+ Mimic+ 0.1uM
Reporter+ Effecter+ Mimic 0.01uM
No DNA

52/56

Fig.40.

Spodoptera exigua DNA sequence.

Sequence ID 6

SPODOPTERA EXIGUA HINGE AND LIGAND BINDING DOMAINS

```
          3           9          15          21          27          33          39          45
          |           |           |           |           |           |           |           |
  1 AGG CCG GAG TGC GTG GTG CCA GAA AAC CAG TGT GCA ATG AAA AGG
    TCC GGC CTC ACG CAC CAC GGT CTT TTG GTC ACA CGT TAC TTT TCC

 46 AAA GAG AAA AAG GCA CAA AGG GAA AAA GAC AAG TTG CCA GTC AGT
    TTT CTC TTT TTC CGT GTT TCC CTT TTT CTG TTC AAC GGT CAG TCA

 91 ACA ACG ACA GTG GAT GAT CAC ATG CCT CCC ATT ATG CAG TGT GAT
    TGT TGC TGT CAC CTA CTA GTG TAC GGA GGG TAA TAC GTC ACA CTA

136 CCA CCG CCT CCA GAG GCC GCA AGA ATT CAC GAG GTG GTG CCA CGA
    GGT GGC GGA GGT CTC CGG CGT TCT TAA GTG CTC CAC CAC GGT GCT

181 TTC CTG AAT GAA AAG CTA ATG GAC AGG ACA AGG CTC AAG AAT GTG
    AAG GAC TTA CTT TTC GAT TAC CTG TCC TGT TCC GAG TTC TTA CAC

226 CCC CCT CAC TGC CAA CCA GAA GTC CTT AAT AGC GAG GCT GGT CTG
    GGG GGA GTG ACG GTT GGT CTT CAG GAA TTA TCG CTC CGA CCA GAC

271 GTA CCA AGA AGG CTA TGA ACA GCC ATC AGA AGA GGA TCT AAA AAG
    CAT GGT TCT TCC GAT ACT TGT CGG TAG TCT TCT CCT AGA TTT TTC
```

Fig.40 i.

```
316 AGT CAC ACA GTC GGA TGA AGA CGA AGA AGA GTC GGA CAT GCC GTT
    TCA GTG TGT CAG CCT ACT TCT GCT TCT TCT CAG CCT GTA CGG CAA

361 CCG TCA GAT CAC CGA GAT GAC GAT CCT CAC AGT GCA GCT CAT TGT
    GGC AGT CTA GTG GCT CTA CTG CTA GGA GTG TCA CGT CGA GTA ACA

406 TGA ATT CGC TAA GGG CCT ACC AGC GTT CGC AAA GAT CTC ACA GTC
    ACT TAA GCG ATT CCC GGA TGG TCG CAA GCG TTT CTA GAG TGT CAG

451 GGA TCA GAT CAC ATT ATT AAA GGC CTG TTC GAG TGA GGT GAT GAT
    CCT AGT CTA GTG TAA TAA TTT CCG GAC AAG CTC ACT CCA CTA CTA

496 GTT GCG AGT AGC TCG GCG GTA CGA CGC GGC GAC AGA CAG CGT GTT
    CAA CGC TCA TCG AGC CGC CAT GCT GCG CCG CTG TCT GTC GCA CAA

541 GTT CGC CAA CAA CCA GGC GTA CAC CCG CGA CAA CTA CCG CAA GGC
    CAA GCG GTT GTT GGT CCG CAT GTG GGC GCT GTT GAT GGC GTT CCG

586 AGG CAT GGC CTA CGT CAT CGA GGA CCT GCT GCA CTT CTG CCG GTG
    TCC GTA CCG GAT GCA GTA GCT CCT GGA CGA CGT GAA GAC GGC CAC

631 CAT GTA CTC CAT GAT GAT GGA TAA CGT CCA CTA TGC ACT GCT CAC
    GTA CAT GAG GTA CTA CTA CCT ATT GCA GGT GAT ACG TGA CGA GTG

676 TGC CAT CGT CAT TTT CTC AGA CCG ACC CGG GCT TGA GCT AAC CCT
    ACG GTA GCA GTA AAA GAG TCT GGC TGG GCC CGA ACT CGA TTG GGA

721 GTT GGT GGA GGA GAT CCA GAG ATA TTA CCT GAA CAC GCT GCG GGT
    CAA CCA CCT CCT CTA GGT CTC TAT AAT GGA CTT GTG CGA CGC CCA
```

Fig.40 ii.

```
766 GTA CAT CCT GAA CCA GAA CAG TCG GTC GCC GTG CTG CCC TGT CAT
    CAT GTA GGA CTT GGT CTT GTC AGC CAG CGG CAC GAC GGG ACA GTA

811 CTA CGC TAA GAT CCT CGG CAT CCT GAC GGA GCT GCG GAC CCT GGG
    GAT GCG ATT CTA GGA GCC GTA GGA CTG CCT CGA CGC CTG GGA CCC

856 CAT GCA GAA CTC CAA CAT GTG CAT CTC ACT CAA GCT GAA GAA CAG
    GTA CGT CTT GAG GTT GTA CAC GTA GAG TGA GTT CGA CTT CTT GTC

901 GAA CGT GCC GCC GTT CTT CGA GGA TAT CTG GGA CGT CCT CGA GTA
    CTT GCA CGG CGG CAA GAA GCT CCT ATA GAC CCT GCA GGA GCT CAT

946 AAA
    TTT
```

Total number of bases is: 948.

Fig.41.

Sequence I.D. 7

Sequence comparison between Heliothis 19R clone and SEcR Taq clone

```
HEcR  RPECVVPENQCAMKRKEKKAQREKDKLPVSTTTVDDHMPPIMQCDPPPPEAARILECVQ
SEcR  RPECVVPENQCAMKRKEKKAQREKDKLPVSTTTVDDHMPPIMQCDPPPPEAARI

HEcR  HEVVPRFLNEKLMEQNRLKNVPPLTANQKSLIARLVWYQEGYEQPSEEDLKRVTQSD
SEcR  HEVVPRFLNEKLMERTRLRNVPPLTANQKSLIARLVWYQEGYEQPSEEDLKRVTQSD

HEcR  EDDEDSDMPFRQITEMTILTVQLIVEFAKGLPGFAKISQSDQITLLKACSSEVMMLR
SEcR  EDEEESDMPFRQITEMTILTVQLIVEFAKGLPAFAKISQSDQITLLKACSSEVMMLR

HEcR  VARRYDAATDSVLFANNQAYTRDNYRKAGMAYVIEDLLHFCRCMYSMMMDNVHYALL
SEcR  VARRYDAATDSVLFANNQAYTRDNYRKAGMAYVIEDLLHFCRCMYSMMMDNVHYALL

HEcR  TAIVIFSDRPGLEQPLLVEEIQRYYLNTLRVYILNQNSASPRGAVIFGEILGILTEI
SEcR  TAIVIFSDRPGLELTLLVEEIQRYYLNTLRVYILNQNSRSPCCPVIYAKILGILTEL

HEcR  RTLGMQNSNMCISLKLKKRKLPPFLEEIDWDV
SEcR  RTLGMQNSNMCISLKLKNRNVPPFFEDIDWDV
```

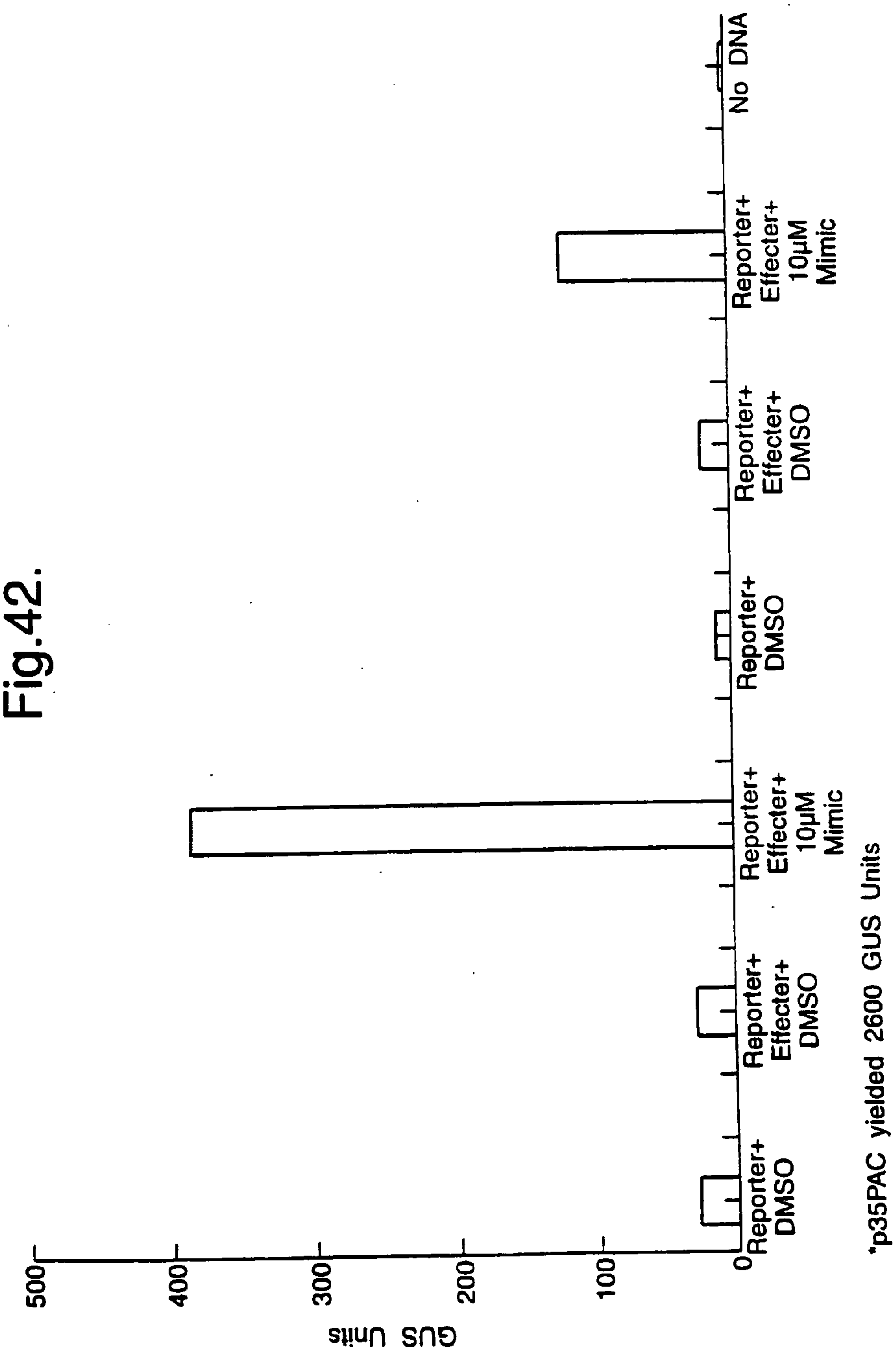

GUS Units
500
400
300
200
100
0
Reporter+ DMSO
Reporter+ Effecter+ DMSO
Reporter+ Effecter+ 10μM Mimic
Reporter+ DMSO
Reporter+ Effecter+ DMSO
Reporter+ Effecter+ 10μM Mimic
No DNA
*p35PAC yielded 2600 GUS Units

Fig.42.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 96/01195

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/12 C12N15/85 C12N15/62 C07K14/72 C07K19/00
C12N5/10 A61K38/16

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K C12N A01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO,A,93 03162 (GENENTECH INC) 18 February 1993	4,5,44, 92-99
Y	see abstract; claims 1-27; figure 1	1,3, 8-43, 45-49, 51-91

X	WO,A,91 13167 (UNIV LELAND STANFORD JUNIOR) 5 September 1991	4,5,44, 50,93-99
Y	see abstract; claims 2,24	2,3

	-/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
9 August 1996	19.08.96

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Gurdjian, D

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	CELL, OCT 4 1991, 67 (1) P59-77, UNITED STATES, XP002010069 KOELLE MR ET AL: "The Drosophila EcR gene encodes an ecdysone receptor, a new member of the steroid receptor superfamily." see the whole document	4,5 1-3, 8-43, 45-49, 51-92
X	INSECT BIOCHEM MOL BIOL, JAN 1993, 23 (1) P115-24, ENGLAND, XP002010070 IMHOF MO ET AL: "Cloning of a Chironomus tentans cDNA encoding a protein (cEcRH) homologous to the Drosophila melanogaster ecdysteroid receptor (dEcR)." see the whole document	4,5
X	INSECT BIOCHEM MOL BIOL, JAN 1995, 25 (1) P19-27, ENGLAND, XP002010071 CHO WL ET AL: "Mosquito ecdysteroid receptor: analysis of the cDNA and expression during vitellogenesis." see the whole document	4,5,52, 53
Y	EP,A,0 615 976 (AMERICAN CYANAMID CO) 21 September 1994 see page 6, line 28 - line 32; claims 1-12; example 2	8-43, 45-49, 51-92
Y	EUR. J. ENTOMOL. (1995), 92(1), 333-40 CODEN: EJENE2;ISSN: 1210-5759, XP002010346 SMAGGHE, GUY ET AL: "Biological activity and receptor -binding of ecdysteroids and the ecdysteroid agonists RH-5849 and RH-5992 in imaginal wing discs of Spodoptera exigua (Lepidoptera: Noctuidae)" see page 336, paragraph 3 - page 337, paragraph 2	51-65
A	DEVELOPMENTAL GENETICS, 1995, 17, 319-330, XP002010345 KOTHAPALLI R ET AL: "CLONING AND DEVELOPMENTAL EXPRESSION OF THE ECDYSONE RECEPTOR GENE FROM THE SPRUCE BUDWORM, CHORISTONEURA-FUMIFERANA" see the whole document	1-5, 51-54

-/--

4

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	INSECT BIOCHEM. MOL. BIOL. (1994), 24(8), 763-73 CODEN: IBMBES;ISSN: 0965-1748, XP002010072 JINDRA, MAREK ET AL: "Isolation and developmental expression of the ecdysteroid-induced GHR3 gene of the wax moth Galleria mellonella" see the whole document ---	1-5
A	US,A,5 424 333 (WING KEITH D) 13 June 1995 see column 150, paragraph 3 - paragraph 7; example 3 -----	97,98

4

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/GB 96/01195

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9303162	18-02-93	EP-A-	0598011	25-05-94
		JP-T-	7501928	02-03-95
WO-A-9113167	05-09-91	AU-B-	1779295	14-09-95
		AU-B-	7492291	18-09-91
		CA-A-	2076386	27-08-91
		EP-A-	0517805	16-12-92
		US-A-	5514578	07-05-96
EP-A-0615976	21-09-94	CA-A-	2112445	01-07-94
		JP-A-	6253849	13-09-94
US-A-5424333	13-06-95	US-A-	5354762	11-10-94
		US-A-	4985461	15-01-91
		AU-B-	637573	03-06-93
		AU-B-	3636089	21-12-89
		DE-D-	68909548	04-11-93
		DE-T-	68909548	10-03-94
		EP-A-	0361645	04-04-90
		ES-T-	2059755	16-11-94
		JP-A-	2152922	12-06-90
		AU-B-	628349	17-09-92
		AU-B-	3645489	21-12-89
		DE-D-	68908789	07-10-93
		DE-T-	68908789	14-04-94
		EG-A-	18874	30-06-94
		EP-A-	0347216	20-12-89
		ES-T-	2059754	16-11-94
		IL-A-	90606	31-07-95
		JP-A-	2042049	13-02-90
		PT-B-	90863	31-01-95
		SG-A-	114793	10-06-94
		US-A-	5117057	26-05-92
		AU-B-	595303	29-03-90
		AU-B-	7147387	04-02-88
		CA-A-	1310641	24-11-92
		DE-A-	3774347	12-12-91
		EG-A-	18544	30-01-94
		EP-A-	0253468	20-01-88
		ES-T-	2037709	16-07-96

Form PCT/ISA/210 (patent family annex) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/GB96/01195

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.: 98
because they relate to subject matter not required to be searched by this Authority, namely:
Although this claim is directed partly to a method of treatment of the human/animal body the search has been carried out and based on the alleged effects of the compound/composition

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/GB 96/01195

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US-A-5424333		IE-B- 59964	04-05-94
		JP-B- 7098806	25-10-95
		JP-A- 63023866	01-02-88
		KR-B- 9505199	19-05-95
		AU-B- 602505	18-10-90
		AU-B- 6926687	03-09-87
		CA-A- 1295618	11-02-92
		EP-A- 0234944	02-09-87
		ES-T- 2032818	16-07-96
		KR-B- 9410277	22-10-94
		AU-B- 599970	02-08-90
		AU-B- 7147287	31-03-88
		CA-A- 1331189	02-08-94
		DE-A- 3783111	28-01-93
		EP-A- 0261755	30-03-88
		ES-T- 2053535	01-08-94
		IE-B- 59962	04-05-94
		JP-B- 8005854	24-01-96
		JP-A- 63083063	13-04-88
		KR-B- 9513856	17-11-95
		AU-B- 597912	14-06-90
		AU-B- 6428986	30-04-87